



COMPARATIVE HISTOPATHOLOGY OF  
MUCOSA ASSOCIATED LYMPHOID  
TISSUE IN THE GILLS OF ATLANTIC  
SALMON *SALMO SALAR* AND SOUTHERN  
BLUEFIN TUNA *THUNNUS MACCOYII*

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# Declarations by the Author

## Statement of Originality

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Signed

Catarina do Carmo Norte dos Santos

# Statement of Co-Authorship and Thesis Contributions

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## **Paper 1 (Chapter 2)**

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# Abstract

The recent identification of an interbranchial lymphoid tissue (ILT) revealed that an organized mucosa-associated lymphoid tissue (MALT) was present in the gills of salmonids. Due to its location in the gills, and lymphoid nature, it was hypothesized that the ILT could be affected by amoebic gill disease (AGD). AGD is caused by a protozoan parasite, *Neoparamoeba perurans*. This disease is considered a serious problem for the health and welfare of marine farmed salmonids. AGD affects the gills of fish, causing significant pathology, such as hyperplasia of the lamellae leading to lamellar fusion. This research, therefore, aimed to understand the effects of AGD in the ILT of Atlantic salmon (*Salmo salar*) using histological techniques. Two studies were undertaken to evaluate the effects of AGD in the structure and cellularity of the ILT. In the first study, Atlantic salmon were exposed to *N. perurans* trophozoites, and sampled prior to exposure and at 3, 7, 14 and 28 days post-exposure. Histology was performed for the first experiment, and the area of the proximal ILT and cell density were quantified. It was found that AGD caused changes in the morphology of the ILT, which was enlarged at 28 days post-exposure to *N. perurans* when compared to controls. Proliferating cell nuclear antigen (PCNA) immunostaining revealed that epithelial hyperplasia was the most likely factor contributing to the ILT enlargement in the affected fish. Lymphocyte density in the ILT of affected fish was significantly lower 28 days post-exposure.

In the second study, fish were tagged with passive integrated transponder (PIT) tags, exposed four times to *N. perurans* trophozoites and treated following each exposure in freshwater. This experiment intended to mimic the infection pattern in an aquaculture environment. At the end of the experiment, the effects of exposure time (either single infection or repeated infections) and lesion presence on the ILT were assessed. Changes in the T-cell population were primarily associated with lesion presence. Histological assessments suggested observed changes in the ILT could be indicative of different AGD pathology stages with different cell types present in the ILT.

The presence of organized MALT in the gills of non-salmonid teleosts has not been confirmed. However, it is likely that a similar structure may be present in the gills of other fish species. To investigate this hypothesis, the gills of a Scombridae, the southern bluefin tuna (SBT) (*Thunnus*

*maccoyii*), a species with both high economic and recreational value in Australia, was investigated. Histological observations of the gill indicated the filamental mucosa overlaid a tissue composed mainly of lymphocytes and eosinophilic granulocytes embedded in a meshwork of epithelial cells. A basement membrane underlaid the lymphoid cell accumulation, separating it from the gill connective tissue. To the best of our knowledge, this is the first histological description of a gill associated lymphoid tissue (GIALT) in the gills of SBT.

Overall, the results of this research have provided important information regarding the ILT during AGD infection in naïve and Atlantic salmon repeatedly exposed to *N. perurans*. It was found that the ILT is a plastic tissue, changing its size and shape during AGD. Additionally, a difference in the cell population of the ILT was related to AGD severity. This research is also the first to identify a GIALT in the gills of SBT. The major difference between the lymphoid tissue in the SBT and the ILT in salmonids was the presence of a population of eosinophilic granulocytes in the lymphoid tissue present in the gills of SBT. These results aid in the understanding of the GIALT in both Atlantic salmon and SBT. The information acquired in this project will be useful for further investigation of the ILT during AGD, mucosal immunology and mucosal vaccine development. Furthermore, the characterization of an GIALT in the gills of SBT provides novel information that could relate to the phylogeny of MALTs in vertebrates.

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# Chapter One

## General Introduction

### 1. Fish Immune System

The aquatic environment, having a variable and abundant microbiota, has ideal conditions for the growth of microorganisms (Bergh et al., 1989; Gomez et al., 2013). These conditions challenge the immune system of aquatic vertebrates when compared with their terrestrial counterparts (Gomez et al., 2013). Aquatic vertebrates have an effective immune response and fish are the first animal phyla presenting both innate and adaptive immune system (Magnadottir, 2010). Fish are very different from terrestrial vertebrates especially in the location and distribution of immune relevant cells, tissues, and organs (Cooper, 2001). The most striking example is the lack of bone marrow and lymph nodes, which are first present in amphibians (Cooper, 2001). Despite differences between the immune system among jawed vertebrates, they share immunologically relevant body areas (such as the gut mucosal surfaces) that throughout evolutionary time have been subjected to very similar selective pressures (Sunyer, 2013). Furthermore, the study of the teleost immune system provides essential insight into unresolved paradigms of mucosal immunity in mammals (Sunyer, 2013).

Physiologically, the immune system of fish has similarities to the immune system of higher vertebrates. However, different strategies of immune protection exist as a consequence of the distinct environment and metabolism of fish. Being a free-living organism in early embryonic stages, fish rely on the innate immune response for survival (Rombout et al., 2005). Additionally, fish are constantly immersed in media with potentially harmful microorganisms being held in suspension (Magnadottir, 2010; Tort et al., 2003). Thus, the innate immune response is of high importance for fish, due to the limitations of the adaptive immune response which has a limited repertoire of antibodies (Whyte, 2007). Acquired immune response consists of lymphocyte populations that are analogous to T-cells, B-cells and cytotoxic cells (similar to natural killer cells), (Manning and Nakanishi, 1996).

Immunocompetent cells develop and respond to antigens in specialized lymphoid tissues which are a type of connective tissue that is characterized by a rich supply of lymphocytes. Lymphoid

tissues are made up of free cells that are supported by a rich network of reticular fibrils, made principally of type III collagen (Abrahamsohn, 2005). These fibrils are produced by the reticular cell, which is a fibroblastic cell. The cells can exist free within the connective tissue, or comprise a lymphoid organ surrounded by a capsule (Abrahamsohn, 2005).

## 1.1 Mucosa-Associated Lymphoid Tissues

Mucosal areas are a thin epithelial barrier that facilitates several processes that are crucial for maintaining homeostasis, such as gas exchange and acid-base regulation and excretion. Furthermore, mucosal surfaces have developed to optimize their function (Evans et al., 2005; Salinas and Miller, 2015). Despite being a specialized contact point with the external environment, mucosal areas of fish are also constantly exposed to the external milieu, which makes them a likely entry site for pathogens (Salinas and Miller, 2015). However, mucosal surfaces are equipped with active immune sites with a diverse and unique repertoire of innate and adaptive immune cells and molecules (Lazado and Caipang, 2014; Salinas, 2015). In general, mucosa-associated lymphoid tissues (MALTs) are composed of migratory and non-migratory cell populations, including lymphocytes, monocytes, granulocytes, macrophages and thrombocytes, supported by a reticular cell framework (Press and Evensen, 1999).

## 1.2 Cellular Components of Teleost MALTs

The classification of blood cells in humans is mostly based on morphology, staining affinity and specific enzymes (Mescher, 2010). However, in fish, these methods cannot be applied to some cells, such as granulocytes (Burrows et al., 2001; Claver and Quaglia, 2009; Esteban et al., 2000). Nevertheless, the equivalent to mammalian T and B lymphocytes, eosinophils, macrophages, neutrophils, mast/eosinophilic granule cells, thrombocytes, dendritic cells (in some species) and natural killer cells have been described in fish (Lugo-Villarino et al., 2010; Secombes, 1996; Whyte, 2007). The most significant cells in fish teleost MALTs are T and B lymphocytes, eosinophils, macrophages, neutrophils, mast cells and dendritic cells (Castro and Tafalla, 2015; Gomez et al., 2013b). Furthermore, epithelial cells are also considered important cells in these structures (Gomez et al., 2013b).

## 1.2.1 Cellular components of teleost MALTs-Cells of the innate immune system

### *Epithelial Cells*

Epithelial cells (ECs) are more than the blocks that compose the thin layer that protects the underlying structures from potentially noxious elements (Gomez et al., 2013a). They also orchestrate homeostasis and have an important role in innate and adaptive immune responses (Hill and Artis, 2010). ECs have a phagocytic function that allows them to eliminate pathogens from the integument (Rimoldi et al., 2005). Furthermore, it is known that ECs express major histocompatibility complex (MHC) class I and II (Castro and Tafalla, 2015). In the gills of rainbow trout (*Oncorhynchus mykiss*) the expression of MHC class I was very high in the epithelia covering the lamellae and the filament (Dijkstra et al., 2003). High expression of MHC class I and II was also observed in the gills of Atlantic salmon (*Salmo salar*) (Koppang et al., 2003). It was suggested that the high transcript levels of MHC class II cells in the gills of salmon was due to the abundance of MHC class II positive epithelial cells, rather than an abundance of lymphocytes expressing these molecules (Koppang et al., 2003). The presence of MHC class I and II in ECs indicates that they can activate T-cells that are restricted to recognizing antigens with the presence of MHC class I or II (Bals and Hiemstra, 2004). ECs use pattern recognition molecules to bind to conserved molecular patterns present in pathogens (Bals and Hiemstra, 2004). It has been hypothesized that ECs of the intestine of carp (*Cyprinus carpio*) participate in the transfer of macromolecules to phagocytes (Rombout et al., 1985; Rombout et al., 2011). Furthermore, ECs express polymeric Ig receptor (pIgR), which is a component essential for mucosal immunity, transporting IgT and IgM across mucosal barriers (Gomez et al., 2013a; Rombout et al., 2011).

### *Dendritic Cells*

In mammals, dendritic cells (DCs) are considered the most important antigen-presenting cell, with the widest range of antigen presentation measures (Salinas, 2015). Additionally, they regulate the differentiation of T-cells into regulatory T-cells (Tregs) by secreting specific cytokines (Salinas, 2015). Furthermore, in mammals, DCs have the capacity to imprint mucosal

homing molecules specifically on B and T-lymphocytes (Mora et al., 2003; Mora et al., 2006). In teleosts, DCs have been characterized in zebrafish (*Danio rerio*) and salmonids (Haugland et al., 2012; Lovy et al., 2009; Lugo-Villarino et al., 2010). DCs in mammals commonly express major MHC class II, and can be up or down-regulated by several external factors (Glimcher and Kara, 1992). Cells positive for this protein and with a similar structure to mammal DCs have been identified in the filamental and lamellar epithelium of salmonids (Koppang et al., 2003; Olsen et al., 2011). The nature of DC populations in teleosts however will remain unclear until accurate tools such as specific cell markers are developed for more in-depth study.

#### *Monocytes and Macrophages*

Monocytes are the precursor of macrophages, which are the main phagocytic cells of the immune system, and are present in all teleost MALTs (Castro and Tafalla, 2015). They are mainly present in the tissue throughout the body and are quickly recruited upon tissue damage or infection (Castro and Tafalla, 2015). The main function of macrophages is to maintain homeostasis and balance with the commensal microbiota (Gomez et al., 2013b). In fish, four different phenotypes of macrophages have been described (Forlenza et al., 2011). Innate activated macrophages are induced by a microbial stimulus and activate the macrophages to become phagocytic (Castro and Tafalla, 2015). The classically activated macrophages are induced by a combination of microbial stimulus and Interferon (IFN)  $\gamma$  (Forlenza et al., 2011). Both, activation and function of these types of macrophages are very different among teleost species (Forlenza et al., 2011). The alternatively activated macrophages require the presence of the cytokines IL-4 and/or IL-13 (belonging to the T-helper 2 pathway [Th2]) and mostly act as an anti-inflammatory macrophage (Castro and Tafalla, 2015). The regulatory macrophages are associated with IL-10, an anti-inflammatory cytokine, and are reported to be involved in the down-regulation of inflammation (Castro and Tafalla, 2015).

#### *Polymorphonuclear Leukocytes*

The polymorphonuclear leukocytes such as neutrophils, eosinophils, and mast cells are a controversial topic in fish immunology. This is because the nomenclature and classification methods used in mammals do not always correlate with the characteristics of these cells observed in fish (Ainsworth, 1992).

Neutrophils are phagocytic cells that are the first to be recruited to sites of inflammation (Castro and Tafalla, 2015). They have a strong antimicrobial activity by degranulation of cytoplasmic granules releasing a vast assortment of antimicrobial peptides, redox molecules, and enzymes. Neutrophils also induce the activation and maturation of immune cells through signaling (Castro and Tafalla, 2015).

Eosinophils have an important role in the immune response against internal parasites and also in the modulation of immune response in mammals. In southern bluefin tuna (SBT) (*Thunnus maccoyii*) the most common granulocytes in circulation are eosinophils (Rough et al., 2005). Infiltration of eosinophils in gill areas of Atlantic bluefin tuna (*Thunnus thynnus*) affected by the parasite *Didymosulcus katsuwonicola* has been described (Pleic et al., 2015). Furthermore, eosinophils have been successfully isolated from the whole kidney marrow and thymus of zebrafish using a transgene of Gata-2, which is used to differentiate and maintain murine eosinophils (Balla et al., 2010).

Cells that structurally and functionally resemble mammalian mast cells have been described in most teleosts (Castro and Tafalla, 2015). It has been suggested that teleost mast cell precursors produced in the hematopoietic system are released into the blood where they undergo the maturation process (Gurish and Boyce, 2006; Hallgren and Gurish, 2011). Furthermore, the recruitment of mast cells to sites where parasitic infections or tissue lesions occurred, indicates a general role in persistent inflammatory reactions in teleosts (Castro and Tafalla, 2015). Other populations of innate immune granulocytes that have been reported to participate in the elimination of parasites are the rodlet cells (Leino, 1996; Manera and Dezfuli, 2004). When a parasite attaches to an epithelial surface, rodlet cells are recruited and accumulate in the affected area (Reite and Evensen, 2006). These cells have a distinctive structure, with cytoplasmic inclusions that possess a crystalline inner core (Castro and Tafalla, 2015).

## 1.2.2 Cellular Components of Teleost MALTs-Cells of the Adaptive Immune System

The adaptive immune system emerged when Agnathans appeared, and it has been hypothesized that microbial colonization of mucosal surfaces has driven the appearance of this type of immune response (Lee and Mazmanian, 2010). The need for distinguishing between self and foreign

antigens is difficult when the mucosal surface is colonized by a high burden of microbial symbionts (Lee and Mazmanian, 2010). The innate immune system does not have the capacity to distinguish the symbiont microorganisms from the pathogens because both have similar molecular patterns, such as toll-like receptors ligands. Thus, it is hypothesized that immune tolerance (rather than ignorance) is what occurs in mucosal surfaces (Lee and Mazmanian, 2010).

The adaptive immune system is composed mainly of two lineages of lymphocytes, the T (for thymus-derived) and B (for bursa or bone-marrow-derived) lymphocytes (Cooper and Alder, 2006). One important characteristic of these cells is the presence of antigen binding regions named T-cell receptors (TCRs), and B-cell receptors (BCRs) (Cooper and Alder, 2006). Both receptors share a common ancestor, having similar domain organization and both depend on the same rearrangement mechanisms to generate diversity (Davis et al., 1984; Flajnik and Kasahara, 2010).

B and T-cells present structurally diverse antigen receptors for the recognition of specific pathogens (Castro and Tafalla, 2015). In contrast to TCR, which are always connected to the cell surface (Castro and Tafalla, 2015), BCRs are exposed on the surface and are secreted as immunoglobulin (Ig) or as antibodies (Janeway et al., 2001). Immunoglobulins are present in body fluid such as serum and mucosal secretions, and are mainly produced by plasmablasts and plasma cells (Salinas et al., 2011). They can be present in a soluble form (antibodies), or in the surface of B-cells as B cell receptors (Salinas et al., 2011). Teleost B-cells express three classes of immunoglobulins: IgM, IgD (Warr et al., 1979; Wilson et al., 1997) –and the recently discovered IgT and IgZ isotype (Hansen et al., 2005; Danilova et al., 2005). Hansen et al. (2005) described an IgT isotype in rainbow trout, whereas Danilova et al. (2005) described an IgZ isotype in zebrafish. In rainbow trout affected by a protozoan parasite (*Ceratomyxa Shasta*) IgM was the most prevailing isotype in the serum, whereas the concentration of IgT was double in the gut mucus than in the serum (Zhang et al., 2011). This suggests that IgT may have an important role in the mucosa of teleosts. In rainbow trout, it has been proposed that IgT may have anti-inflammatory properties, which are critical for the mucosal homeostasis (Zhang et al., 2011).. B-cells carry unique Ig domain-containing receptors. Contact with only one specific antigen will

induce a clone of cells that will react specifically with that particular antigen (Janeway et al., 2001).

T-cells can be divided into two populations, distinguished by the expression of the membrane-bound glycoproteins, which is also called a cluster of differentiation (CD). Cytotoxic T-cells, which express CD8 kill tagged or infected cells, interact with MHC class I (Alberts et al., 2002). The T-helper (Th) cells that express CD4 in the surface membrane regulate other cells by releasing cytokines (Boschi et al., 2011; Fischer et al., 2013). After stimulation induced by engagement of TCRs to a pathogen, master regulators will orchestrate the differentiation of effector CD4<sup>+</sup> T-cells into Th1, Th2, Th17 or Treg lineages (Castro et al., 2011). The different subsets are characterized by the pattern of cytokine secretion. Two of the T-cell subtypes which demonstrate a very high degree of plasticity are the Th17 and Treg cells due to their capacity to produce cytokines that could be considered hallmarks of opposite lineages (Zhou et al., 2009). A further study suggested that the coordination of both immune responses of these two subtypes (Th17 and Treg) was beneficial to both the host and the microbiota (Lee and Mazmanian, 2010).

## 1.3 Humoral Components Present in MALTs

Humoral immunity is a very important part of the immune response, and it includes cellular receptors or molecules that are present in body fluid components (Uribe et al., 2011). These factors not only provide protection against pathogens but also orchestrate the immune mechanisms, contributing to homeostasis (Castro and Tafalla, 2015).

Cytokines are a superfamily of proteins that mediate cell signalling between other immune cells (Cameron and Kelvin, 2000). They are released mainly by leucocytes and in regulate cells in two possible ways: paracrine, when interacting with a specific receptor on another cell, or autocrine, when interacting with the cell receptors of the cell that releases it (Zhang and An., 2007).

Cytokines can be divided according to the type of signalling. Interleukins are molecules that are involved in the intercellular regulation of the immune system (Zhang and An., 2007).

Interleukins are produced in different cells, but mainly in T-helper cells (CD4<sup>+</sup>), macrophages/monocytes and endothelial cells (Secombes et al., 2011). Interleukins can be pro-inflammatory or can act as an inhibitor to the activities of other interleukins (Secombes et al., 2011). There are four main Interleukin families. The Interleukin-1 (IL-1) is considered a family

of pro-inflammatory Interleukin, even though some members can be a regulator of other interleukins from the same family (Dinarello, 2009). The Interleukin-2 (IL-2) family is important for the maintenance of T-cell memory (Osborne and Abraham, 2010; Secombes et al., 2011). The members of the Interleukin-10 (IL-10) subfamily are considered anti-inflammatory cytokines and have been reported to be induced in fish species during bacterial infections (Inoue et al., 2005; Pinto et al., 2007; Seppola et al., 2008). The IL-17 subfamily is involved in the inflammatory and neutrophil response (Benedicenti et al., 2015). Chemokines regulate the trafficking of immune cells, orchestrating not only leucocyte transit but also micro-environmental segregation within lymphoid organs (Cyster, 1999; Warnock et al., 2000). IFN are induced by viruses and have antiviral activity (Zou and Secombes, 2011). In mammals IFN can be divided into three families: IFN I IFN II and IFN III. IFN I and orthologs of IFN II are present in several teleost species as reviewed in Zou and Secombes (2011).

Complement is considered a multicomponent defence system, with several functions, including induction of inflammatory response, elimination and clearance of pathogens and cell debris, and modulation of immune responses (Magnadottir et al., 2005; Walport, 2001). Small peptides named antimicrobial peptides (AMPs) are very important components of the innate immune response, directly leading to the lysis of pathogens. AMPs also have an important role in neutralization of endotoxins, chemotactic activity and immunomodulation (Castro and Tafalla, 2015).

## 1.4 The Anatomy of MALTs

Mucosal-associated lymphoid tissues (MALTs) can be subdivided according to their morphology and functionality. The diffuse MALT is composed of widespread lymphocytes scattered throughout the mucosal areas (Montilla et al., 2004; Salinas, 2015). Organized MALTs evolved from diffuse MALTs which consist of mucosal follicles that are responsible for the induction phase of the immune response (Montilla et al., 2004; Salinas, 2015). Organized MALTs provide niches where the selection of high-affinity B-cell clones occurs. Thus, it is believed that organized MALTs have provided the basis for the maturation of antibody responses (Salinas, 2015).



Teleost MALTs are composed of scattered populations of leukocytes along the skin (SALT), intestine (GALT) and nasopharynx (NALT) (Salinas, 2015). The only known organized MALT in teleosts is the recently described interbranchial lymphoid tissue (ILT) which is part of the gill-associated lymphoid tissue (GIALT) (Haugarvoll et al., 2008).

## 1.5 Gill-Associated Lymphoid Tissue (GIALT)

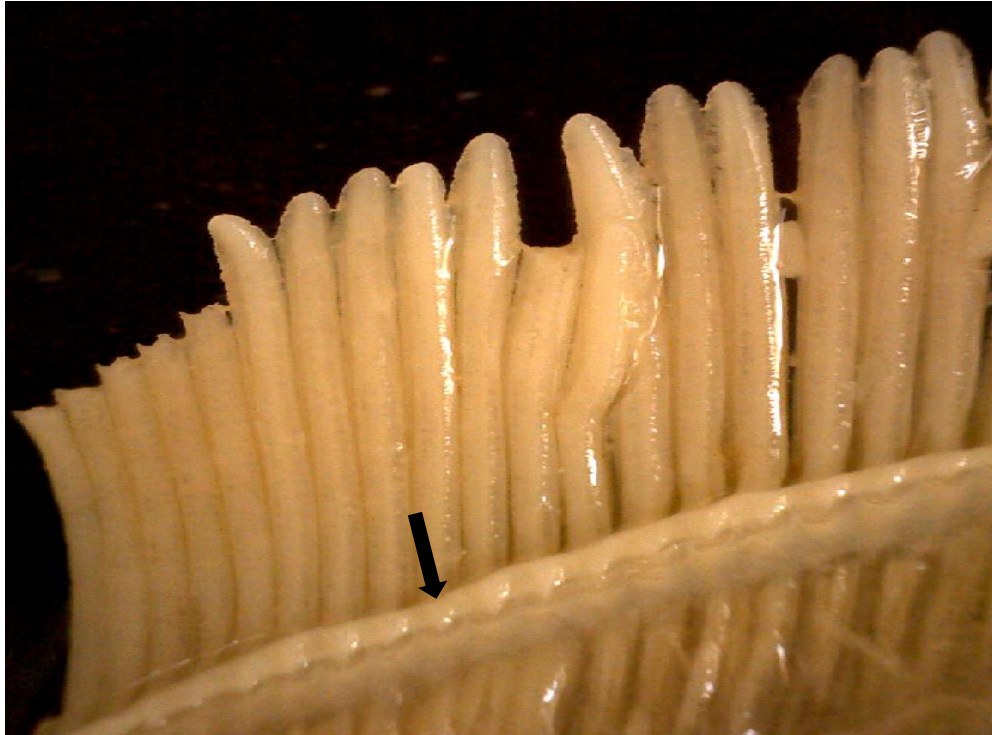
Fish gills are considered the most physiologically diversified organ in vertebrates (Olson, 2002). They are the primary if not sole organ of respiration, osmoregulation, and nitrogen excretion and also major contributors to acid-base balance and hormone metabolism (Olson, 2002). Gills are formed by epithelium, glycocalyx, and a mucous layer. These layers represent the physical barriers between the gills and the external environment (Powell et al., 1994). Despite the physical barrier, gills are an obvious entry point for pathogens (Haugarvoll et al., 2008). Thus, several studies focusing on the immune response in the gill have found that gills are an important organ for innate and acquired immune responses (Rombout et al., 1989). The innate and acquired immune responses in gills have been supported by the capacity to take up antigen (Rombout et al., 1989; Zapata et al., 1987) and the presence of a distinct mucosal IgM isotype and cells secreting this isotype in gills and intestine (Rombout et al., 1993). MHC class II  $\beta$  chain expression was also detected in the gills of Atlantic salmon (Koppang et al., 1998). The gills of fish continuously express immune factors that represent an important protection mechanism against virus invasion. These are Mx proteins, which belong to the class of dynamin-like large guanosine triphosphatases (GTPases), that contribute to intracellular vesicle trafficking as well as organelle homeostasis (Jensen et al., 2002, Haller et al., 2007). Toll-like receptor 9 and T-cell receptor (TCR) transcripts are also expressed in the gills of Japanese flounder (*Paralichthys olivaceus*) (Takano et al., 2004; Takano et al., 2007) and MHC class II  $\beta$  chain was found to be at relatively higher levels in the gills of Atlantic salmon than in the lymphoid pronephros and spleen, and expression could be induced after intraperitoneal vaccination against pathogens (*Aeromonas salmonicida* ss. *salmonicida*, *Vibrio anguillarum* and *Vibrio salmonicida*) (Koppang et al., 1998). Homologues of the dendritic cell marker CD83, which regulates lymphocyte activation, maturation and homeostasis of T and B-lymphocytes, was cloned and sequenced from the gills of the nurse shark (*Ginglymostoma cirratum*) and rainbow trout (Ohta et al., 2004). Koppang et al. (2003) reported that immunoreactive gill epithelium cells were present in the gills

of Atlantic salmon. Further, studies in Atlantic salmon revealed that lymphoid-like tissue aggregates observed at the base of the gill filaments were highly reactive. Haugarvoll et al. (2008) investigated the likely presence of aggregates of lymphoid cells, and the localization of the lymphoid-like tissue. This investigation led to the finding of an organized mucosal-associated lymphoid tissue, named interbranchial lymphoid tissue (ILT) (Haugarvoll et al., 2008).

### *1.5.1 Interbranchial Lymphoid Tissue*

The ILT was first described by Haugarvoll et al. (2008) as an intraepithelial lymphoid cell aggregation in the interbranchial septum of salmonids. Further analysis found that the majority of the cells were T-lymphocytes (Koppang et al., 2010).

The ILT has been divided into proximal ILT (pILT), located in the very terminal end of the interbranchial septum, and distal ILT (dILT), located along the trailing edge of the lymphoid tissue (Dalum et al., 2015) (Figure 1.1). Both the proximal and distal ILT present very similar cell arrangement consisting of a continuous network of epithelial cells that incorporate a large population of T-cells limited by a basal membrane (Haugarvoll et al., 2008). Both also have very similar immunohistochemical characteristics (Haugarvoll et al., 2008). The ILT is present both in the trailing and leading edge of the filament; however, in the leading edge, it presents low numbers of T-cells and the epithelium is thin (Dalum et al., 2015). Despite similarities between the ILT and the thymus, the ILT does not present compartmentalization analogous to the thymic medulla and cortex (Koppang et al., 2010). It is believed that the ILT may be a secondary lymphoid organ in the early stages of evolution, due to the lack of features, such as vessels and germinal centers (Aas et al., 2014). The recombination activation genes-1 and -2 (RAG-1 and RAG-2) are expressed during the V(D)J (variable, diversity joining) which rely of these proteins to create unique receptors in mature T or B-cells (Huttenhuis et al., 2005). Temporal and spatial patterns of T- and B-cell populations in the common carp can be revealed by the expression of RAG-1 and RAG-2 (Huttenhuis et al., 2005). Although the expression of RAG-1 and RAG-2 can be found in primary lymphoid organs (Hansen and Kaattari, 1995), expression of these genes in the ILT of post-smolt salmonids, was not observed (Aas et al., 2014).

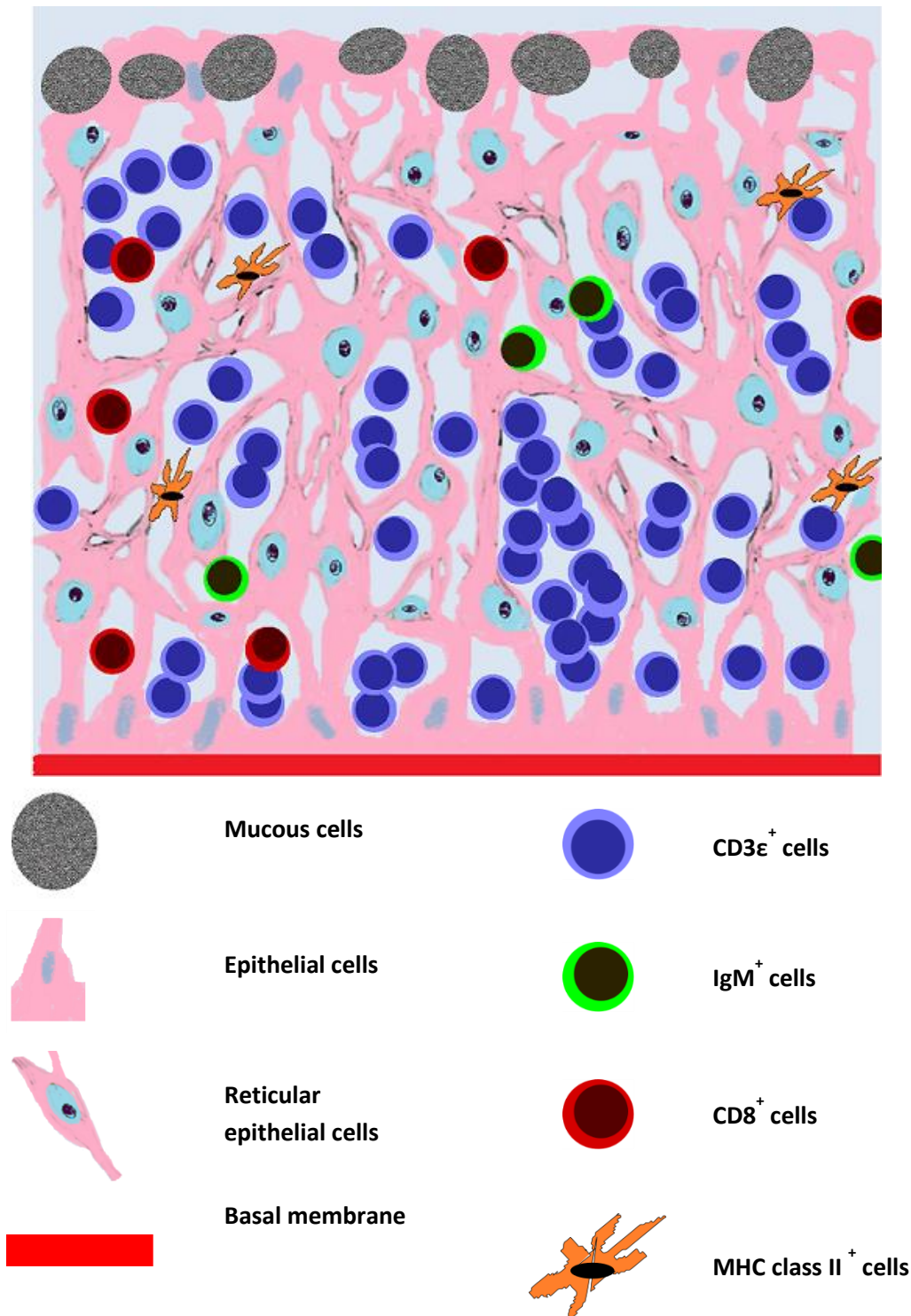


*Figure 1.1 Gill arch sectioned saggittally revealing an hemibranch and the ILT in the central area (arrow)*

#### *Cellular Composition of the ILT*

The ILT is organized in layers (represented in Figure 1. 2). The most external layer is mainly comprised of ovoid mucous cells that contain large apical secretory granules which are prominent in the superficial layer of the epithelium (Dalum et al., 2015). The medial layer is composed mainly of T-lymphocytes ( $CD3\epsilon^+$ ) (Koppang et al., 2010). Transcriptional studies have found a high expression of CD4 genes in the ILT (Aas et al., 2014, Dalum et al., 2015). This may indicate that the majority of the T-cell population in the ILT belongs to the CD4 subset. However, due to the lack of antibodies, the presence of this cell population has not been visualized in the ILT. The  $CD8^+$  cells represented a smaller population of T-cells, and their distribution was limited to the areas that were densely populated by  $CD3^+$  cells (Dalum et al., 2015). MHC class  $II^+$  cells were present in the ILT and even though they represented a small population of the cells present in the ILT, they were uniformly distributed through the epithelium of the ILT and also found in the respiratory lamellas (Dalum et al., 2015). Transcriptional analysis has found that TCR complex is highly expressed in the ILT (Aas et al., 2014). The presence of MHC class II and TCR is important for the T-cell immune response, allowing the

recognition of infected and antigen-presenting cells (Fischer et al., 2013). The presence of IgM<sup>+</sup> cells was found to be sparse in the ILT using specific antibodies (Koppang et al., 2010). However, a transcriptional study found a high expression of IgM in the ILT (Austbo et al., 2014). The explanation for the dissimilar results was attributed to the specificity of IgM antibodies, which only recognize one of the two IgM isotypes in salmonids (Hedfors et al., 2012; Kamil et al., 2011).



*Figure 1. 2 Representation of the structure of the ILT with the superficial layer composed of mucous cells and epithelial cells. The medial structure is composed mostly of CD3 $\epsilon$ <sup>+</sup> cells embedded in a meshwork of epithelial reticular cells. MHC class II<sup>+</sup> cells are present, but the type of cells expressing this receptor is unknown. Scattered CD8<sup>+</sup> cells are present, as well as an unknown number of IgM<sup>+</sup> cells. The ILT is limited by a basal membrane (adapted from Dalum et al., 2015; Haugarvoll et al., 2008).*

### *Immune Response of ILT*

The immune response of ILT has been studied in Atlantic salmon infected with diseases that have a significant effect in aquaculture: Infectious salmon anaemia (ISA), a viral disease, and amoebic gill disease (AGD). AGD is caused by an amphizoic amoeba, *Neoparamoeba perurans*, and has a significant effect in the gills of affected fish (Young et al., 2007). AGD results in focal hyperplasia, which expands along the filament leading to lamellae fusion. Inflammation and oedema are also observed in more advanced stages of the disease (Adams and Nowak, 2003).

Two studies focusing on the ILT of Atlantic salmon infected with ISAV used transcriptional analysis of immune genes in laser-dissected ILT, to ensure that only this tissue was analyzed (Aas et al., 2014; Austbo et al., 2014). Even though no replicating virus was detected in the ILT, an immune response was observed, suggesting that either antigens were presented by local antigen-presenting cells to activate the immune response or a systemic immune response was occurring (Austbo et al., 2014). The immune response of the ILT against ISAV was restrained, when compared with other lymphoid organs (Aas et al., 2014). Thus, it was suggested that similar to mammal lungs, the gills may have a mechanism of regulation which protects this vital organ against severe immunopathological changes (Aas et al., 2014). A previous study found that there was a stronger expression of immunosuppressive receptors and integrins CTLA4 and LAG3 in the T-cells of rainbow trout gills (Takizawa et al., 2011). CTLA4 and LAG3 are related to inhibitory regulation of mucosal T-cells in mammals. This may indicate that the lymphocytes in the mucosal structures have a role in the maintenance of mucosal homeostasis (Takizawa et al., 2011). An up-regulation of IgT genes in the ILT of Atlantic salmon was observed from day 8 of ISAV infection until the final day of sampling and this may represent affinity maturation followed by clonal expansion of IgT expressing B-cells in the ILT (Austbo et al., 2014). Furthermore, a decrease in the size of the ILT of fish infected with ISA-virus was observed, and it was suggested that either lymphocytes were migrating from the ILT to sites exposed to antigens, or shrinkage occurred due to infection (Aas et al., 2014).

A transcriptional analysis of the gill area comprising the ILT in salmon affected by AGD observed a down-regulation of cytokines related to Treg, Th1, and Th17 (Benedicenti et al., 2015). This down-regulation was more evident in fish exposed to high doses of amoeba than those exposed to low doses of amoeba (Benedicenti et al., 2015). The same study reported an up-

regulation of cytokines related to the Th2 pathway, suggesting an immune evasion strategy to avoid cell-mediated killing mechanisms or an allergic mechanism caused by *N. perurans* (Benedicenti et al., 2015).

## 1.4 Research Objectives

The ILT is regarded as an important structure both for possible development of mucosal vaccines, and also evolutionary studies as it is the only organized MALT known in teleosts. However, at this point little is known, and the majority of the studies focus on transcriptional analysis. This research aimed to understand the effects of a protozoan parasite, *N. perurans* on the ILT using histological methods. Understanding the histopathological changes in the ILT caused by a branchial infection with *N. perurans* and subsequent development of AGD will be of high importance to the understanding of the ILT, and for future studies regarding fish mucosal immunity. An additional aim is to determine if the ILT is present in another teleost species, the SBT. If this structure is present in other teleost species, then it may contribute to the understanding of phylogeny of teleost immune structures. Therefore, research in the chapters comprising this thesis has been focused toward completing the following aims:

- Determine the effects of AGD on the structure of the ILT of Atlantic salmon.
  - Investigate the ILT of AGD affected fish sequentially during infection with *N. perurans* by investigating cellular, morphological and morphometric changes during the course of infection.
- Describe the effects of AGD on the CD3<sup>+</sup> cells in the ILT of AGD affected fish.
  - Describe the density of CD3<sup>+</sup> cells in the ILT of Atlantic salmon unexposed, singly and repeatedly exposed to *N. perurans*.
  - Describe the presence of CD3<sup>+</sup> cells in the AGD lesions.
- Investigate the GIALT in another teleost species, the southern bluefin tuna (SBT).
  - Histologically examine the gills of SBT for the presence of a MALT and describe the structure and type of cells where present

# Chapter Two

## Changes in the Interbranchial Lymphoid Tissue of Atlantic Salmon (*Salmo salar*) Affected by Amoebic Gill Disease

Norte dos Santos, C.C., Adams, M.B., Leef, M.J., Nowak, B.F., 2014. Changes in the interbranchial lymphoid tissue of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. Fish & Shellfish Immunology. 41, 600-607.

### Abstract

The interbranchial lymphoid tissue (ILT) was recently described in the gills of salmonids. This study examined changes in the ILT during a parasitic infection in marine environment, using amoebic gill disease (AGD) as a model. Atlantic salmon (*Salmo salar*) experimentally infected with *Neoparamoeba perurans* were sampled at 0, 3, 7, 14 and 28 days post challenge. Transversal sections of three areas of the gills (dorsal, medial and ventral) were histologically assessed for morphological and cellular changes. AGD induced morphological changes and a cellular response in the ILT of affected fish. These changes included a significant increase in the ILT surface area in fish 28 days after AGD challenge, compared to control fish at the same time point. The length of the ILT increased significantly 28 days post exposure in the dorsal area of the gill arch in the fish affected by AGD. The lymphocyte density of the ILT increased after AGD challenge, peaking at 7 days post exposure; however, by 28 days post exposure, a reduction of lymphocyte density to values close to pre-infection levels was observed. PCNA immunostaining revealed that epithelial hyperplasia was the most likely factor contributing to the ILT enlargement in the affected fish.

Keywords: Interbranchial lymphoid tissue; ILT; gill-associated lymphoid tissue; GIALT; T-cell; *Neoparamoeba perurans*; AGD

## 2.1 Introduction



The interbranchial lymphoid tissue (ILT) has recently been described in the gills of salmon. It is located upon the caudal edge of the interbranchial septum at the base of the gill filaments (Haugarvoll et al., 2008). ILT is formed by an accumulation of lymphoid cells, the majority being T cells, supported by a meshwork of interstitial epithelial cells (Koppang et al., 2010). The expression of T-cell receptor (TCR), CD3 $\epsilon$  and MHC class II positive cells together with the organization of the T cells in the center of the ILT and more flattened epithelial cells and mucous cells in the periphery of the ILT indicate an organized lymphoid tissue (Fischer et al., 2013; Haugarvoll, 2008 ). ILT has been considered part of the gill-associated lymphoid tissue (GIALT), combined with dispersed leucocytes within the lamellar epithelium (Salinas et al., 2011). However, being purely an intraepithelial structure makes the ILT different to previously described lymphoid tissues in mammals (Koppang et al., 2010). The majority of the cells present in the ILT were positive for the expression of genes coding for the CD3 $\epsilon$  complex and CD8 co-receptors (Koppang et al., 2010; Takizawa et al., 2011). Those receptors are essential for signal transduction during T cell activation. This starts with the activation of the CD3- signal transduction complex, finishing with the proliferation and differentiation of antigen-specific T cells (Ruddle et al., 2009). Teleost CD8 T cells are typically cytotoxic T cells, which are the principal effectors of cell mediated immune response (Ruddle et al., 2009). Furthermore, the number of Ig<sup>+</sup> cells in the ILT is very low (Koppang et al., 2010). This indicates that the ILT most likely presents a cell-mediated immune function rather than humoral. It has been suggested that immune induction could occur in the ILT, due to the presence of scattered major histocompatibility complex class II (MHC class II) positive cells as well as T-cell receptor (Haugarvoll et al., 2008). A recent study targeting the ILT of salmon infected with salmon anaemia virus (ISAV), showed an immune response against the virus (Austbo et al., 2014). Due to the strategic position regarding antigen surveillance, and the immune response against ISAV, it was suggested that the ILT may have a role as a secondary lymphoid organ with clonal expansion of IgT expressing B-cells (Austbo et al., 2014; Koppang et al., 2010).

Diseases can affect the structure of lymphoid organs of vertebrates (Verburg-Van Kemenade et al., 1999; Secombes and Manning, 1982). Parasitic infections induced changes in the spleen of carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Woo, 1979; Secombes and Manning, 1982). Atlantic salmon infected with ISAV revealed a decrease in size of the ILT (Austbo et al., 2014). In the same study, there was a restrained inflammatory response in the ILT.

It was suggested that, similarly to the mammalian lungs, the gills may have mechanisms of protection against harmful immunopathological changes, perhaps orchestrated by the cells of the ILT (Austbo et al., 2014). To our knowledge, there are no studies that address the effects of marine parasitic conditions on the morphology of the ILT.

Atlantic salmon were experimentally infected with *Neoparamoeba perurans*, the aetiological agent of amoebic gill disease (AGD). This protozoan is a significant threat for several fish species cultured in the marine environment worldwide (Young et al., 2008). The aim of this study was to investigate the effects of *N. perurans* infection in the ILT.

## 2.2 Materials and Methods

### 2.2.1 Challenge

Ethical approval for the conduct of this experiment was granted by the University of Tasmania's Animal Ethics Committee (Permit reference A0012237). Fish rearing and infection were conducted according to (Adams et al., 2012), with changes in the concentration of trophozoites, which was higher in the present study than in the above mentioned. Atlantic salmon (*Salmo salar*) (n=100) from a single cohort of smolt from Saltas hatchery (Wayatinah, Tasmania) (mean initial weight=565g, naïve to AGD) were transferred to two 4000L independent experimental recirculating systems (50 in each tank). Each system had independent mechanical filtration, biofiltration, foam fractionation and UV disinfection. The water quality was checked daily, and maintained at > 90% dissolved oxygen, < 1.0 mg total ammonia, < 5 mg L<sup>-1</sup> NO<sub>2</sub><sup>-</sup>, < 40 mg L<sup>-1</sup> NO<sub>3</sub><sup>2-</sup>, pH 8.0-8.2, temperature of 16.5 ± 0.5 °C and salinity between 33 and 35 ‰. Water changes of approximately 50% system volume were performed every 5 days and the fish were fed twice a day on a commercial feed approximately 1.5% of the bodyweight per day, with no accurate measurement of daily feed intake. The fish were acclimated to seawater (35 ‰) with temperature adjusted to 15°C (± 0.5°C) for 3 weeks prior the challenge. Prior to the introduction of *N. perurans*, 20 fish were sampled randomly from both tanks and their weights and lengths were measured and mucus samples were collected. This first sampling was considered day 0 (pre-infection). After sampling, the fish from one tank were exposed to trophozoites of *N. perurans*, which were isolated from AGD-affected fish according to (Morrison et al., 2004). Trophozoites of *N. perurans* (500 cells L<sup>-1</sup>) were added to one of the systems whilst the filtration

and water flow were disabled for 8 hours. The other system did not have any trophozoites added, but was managed in the same way as the infection system.

## 2.2.2 Sampling

Fish were sampled at 3, 7, 14 and 28 days post exposure (DPE), 6 to 9 fish per time point (Table 2. 1.). The fish captured from the systems on day 0 and 3 were not sedated prior to capture by dip net. The fish collected on days 7, 14 and 28 were briefly sedated ( $13.5 \text{ mg/L}^{-1}$  eugenol) to enable easy capture. Fish were then placed in a 100L tank containing 40L of water and anaesthetic (eugenol:  $30 \text{ mg.L}^{-1}$ ). The weight and length of the fish were recorded, and the gill basket was excised, rinsed in  $0.2 \mu\text{m}$  filtered seawater and then placed into seawater Davidson's fixative. After 24 h in the fixative, the gills were transferred to 70% ethanol until processed. The first left gill arch of both AGD infected and control fish was divided into three sites, where transversal sections were cut: dorsal, medial and ventral. The three transversal gill sections were placed in the same paraffin block and then transferred to the glass slide in a defined order for future reference. The second left gill arch was sectioned longitudinally for the assessment of AGD severity. The samples were processed by routine histology.

*Table 2. 1. Number of replicates (number of fish) analysed per time point and treatment in which three sections of the gill arch were visible and appropriate for performing measurements.*

	Days post exposure				
	0	3	7	14	28
AGD	7	8	8	8	6
Control	7	8	9	7	6

## 2.2.3 Histology and Image Analysis

Sections were cut ( $5 \mu\text{m}$ ) and stained with hematoxylin and eosin (H&E). Samples were then viewed using an Olympus BH-2-RFCA (Japan) compound light microscope. Due to sectioning constraints, just the histological slides where the three sites of the gill arch were visible and image analysis performed was considered for further investigation. For analysis of lymphocyte density in the ILT, two pictures at 1000x magnification were taken per section examined ( $\text{area} = 100 \mu\text{m}^2$ ).

For consistency, the pictures were taken in the same areas of the gill, one in the basal area and the other in the outer area (tip) of the ILT. Lymphocyte numbers were quantified manually using image analysis software (ImageJ - Kurt De Vos, University of Sheffield, Academic Neurology <http://imagej.nih.gov/ij/plugins/cell-counter.html>). Lymphocyte density was defined as the number of lymphocytes per 100  $\mu\text{m}^2$ . The area and length of the ILT were measured from pictures of the transversal sections taken at 100X magnification. The program Image Processing and Analysis in Java (ImageJ) (National Institutes of Health, Bethesda, Maryland, USA) was used for the measurements of the ILT, as follows. After setting a scale according to the image magnification (100X), the polygon tool was used to outline the ILT, and the area was measured by the image analysis software. The length was measured as the distance between the base and the tip of the ILT. To compensate for the size of the fish, the length of the pILT was divided by the log of the weight +1. The area of the ILT was measured in  $\text{mm}^2$  and the length in mm. All the measurements were conducted in the 3 gill sites to determine the effect of the gill site. For the treatment and day post exposure, the average of the three sites was used. The sagittal sections were assessed for AGD severity. The number of well-orientated filaments (the central sinus was visible in at least two-thirds of its length) (Adams and Nowak, 2003) was counted, as well as the number of filaments with hyperplastic lesions. The severity index was calculated as the percentage of affected filaments [(number of filaments with lesion/ total number of filaments) \*100] according to Adams and Nowak (2001).

### *2.2.4 Immunohistochemistry*

Sections from the gills of three AGD affected fish and three controls sampled at both 14 and 28 DPE were selected for proliferating cell nuclear antigen (PCNA) immunostaining. Sections (5  $\mu\text{m}$ ) from the above-mentioned samples were cut and mounted on poly-L-lysine-coated slides (Polysine<sup>TM</sup>, Menzel-Gläser, Germany). The slides were dewaxed and rehydrated, immersed in citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and microwaved for 12 minutes to facilitate heat induced epitope retrieval (HIER), and then left to stand for 20 minutes. The slides were subsequently washed in phosphate buffered saline (PBS). The immunohistochemical identification of proliferating cells was done using an EnVison<sup>TM</sup> G|2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red) kit (Dako corp., Carpinteria, CA, USA). The sections were circled with a PAP pen, rinsed phosphate-buffered saline (PBS), and left to stand in PBS

for 5 minutes. Then, sections were blocked with Dual endogenous block for 5 minutes and rinsed and left to stand in PBS. For identification of proliferating cells, the monoclonal PCNA antibody (1:700, Sigma-aldrich, NSW, Australia) was added, and incubated in a humid chamber for 30 minutes. The slides were rinsed and left to stand in PBS for 5 minutes. A polymer/peroxidase (HRP) was added to the slides for 10 minutes. After that time, the slides were rinsed and left to stand in PBS for 5 minutes. After that a DAB+ working solution was added for 5 minutes. Slides were rinsed with deionized water to stop the reaction then rinsed and left to stand for 5 minutes in PBS. Sections were counterstained with hematoxylin (5 seconds), rinsed quickly with tap water and then dehydrated in 80%, 90% and 100% ethanol concentrations (3 minutes each). The slides were cleared in xylene and mounted. All PCNA positive cells were considered proliferating cells. Positive controls PCNA staining were the gills where an AGD lesion was observed (Adams and Nowak, 2003). Omission of the primary antibody in an additional section for each staining procedure served as the negative control.

### *2.2.5 Data Analysis*

There was no significant difference for fish weight over the duration of the experiment ( $F=1.102$ ,  $df=4,64$ ,  $P=0.363$ ), between treatments ( $F=3.315$ ,  $df=1,64$ ,  $P=0.073$ ) or for the interaction of both factors (day\*treatment) ( $F=0.448$ ,  $df=4,64$ ,  $P=0.773$ ). Thus, no data transformation was made to compensate for the allometric relationship between the body mass of the fish and gill surface area, which becomes significant where size differences between fish approach an order of magnitude (Hughes, 1984). The results for density, area and length of the ILT were analyzed using a 3-way analysis of variance (ANOVA). The main effects analyzed were treatment (2 levels), day (5 levels) and gill site (3 levels). Assumption of normality was checked with the Shapiro-Wilk test and the homogeneity of data was analyzed using the Levene's test. A  $p$  value of  $< 0.05$  was adopted for the rejection of the null hypothesis but when the assumptions failed, the significance level was lowered to 0.01 (Underwood, 1981). When a 3-way interaction was found, to facilitate further analysis and graphing, the data were analyzed separately for each gill site, using a 2-way ANOVA. When the result was statistically significant, the values of the means were compared using a Tukey's honest significant difference (HSD). A regression analysis was used to assess the relationship between AGD severity and the area and length of the ILT of infected fish. A qualitative analysis was made for the sections stained

immunohistochemically for PCNA. Data analysis in PCA stained sections was not done due to the low number of replicates. All statistical analyses were performed with SPSS ® (IBM SPSS Statistics 20, SPSS Science).

## 2.3 Results

### 2.3.1 Histopathology

AGD infection was confirmed by histological analysis of sagittal sections of gill arches from corresponding fish. AGD severity increased with days post exposure (Figure 2. 1). In the samples analysed for infected fish at 3 DPE, 87.5% had visible lesions in at least 1 of the gill sites (dorsal, medial or ventral). At 7 DPE 62.6% fish had lesions in 1 to 3 of the gill sites. The fish samples at 14 and 28 DPE presented 100% of sections with lesions, ranging from 1 to 3 gill sites. The ILT of control fish presented a normal structure, with a network of reticular epithelial cells forming a mesh-like structure enclosing populations of lymphocytes (Figure 2. 2, A,B). There was substantial individual variability in the structure of the ILT of infected fish. Structural changes in the ILT were more evident in the gills of the fish in the later stages of infection (14 and 28 DPE). Those changes were characterized by tissue remodeling of the ILT, assuming a larger and more disorganised morphology when hyperplastic lesions were evident in the area of the filament adjacent to the ILT (Figure 2. 2, C). Furthermore, there was a change in the cellularity of the ILT. Lymphocytes were less abundant, and epithelial cells were more noticeable (Figure 2. 2, D). Association of trophozoites with the ILT was observed in two infected fish sampled on 14 DPE. In these cases, the ILT was enlarged and disorganised to a larger extent than the ILT in sections where lesions were observed in the area close to the ILT (Figure 2. 2, E). Remodeling of the cellular structure of the ILT was observed in the sections where trophozoites were associated with the ILT and in some sections where lesions were found in close proximity to the ILT. In these sections the ILT presented a more pronounced epithelial arrangement and with the increased presence of epithelial cells, there was a depletion of lymphocytes (Figure 2. 2, F). PCNA A staining revealed that there was proliferation of cells in both infected and control fish, however, in AGD affected fish 14 and 28 DPE most of PCNA positive cells had an elongated shape, suggesting that the majority of proliferating cells were epithelial cells epithelial (Figure 2. 3, A, B, C, D).

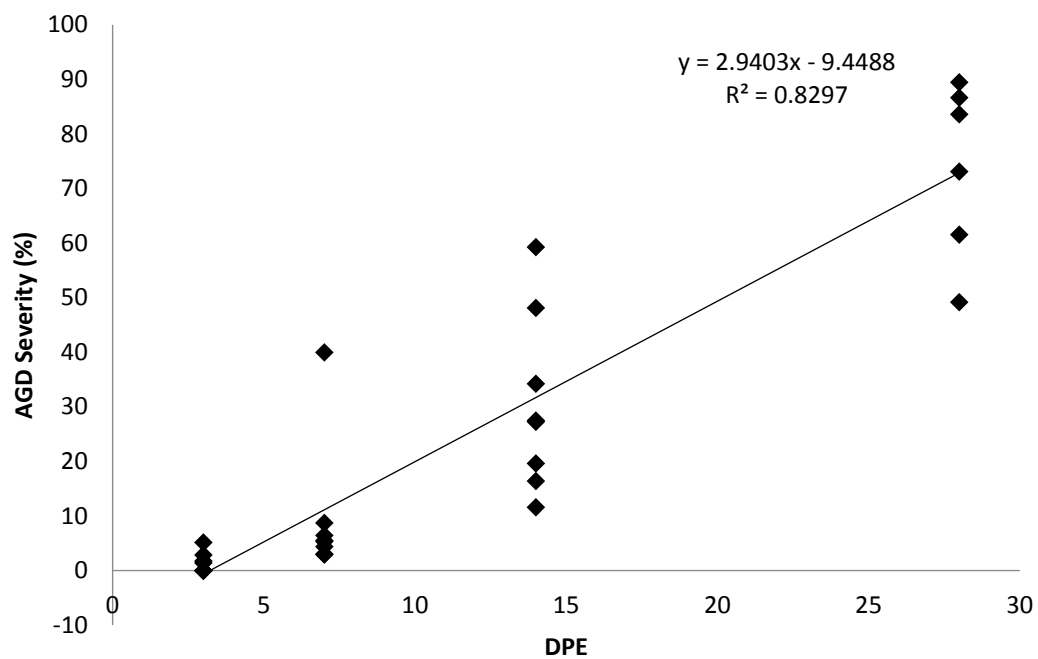
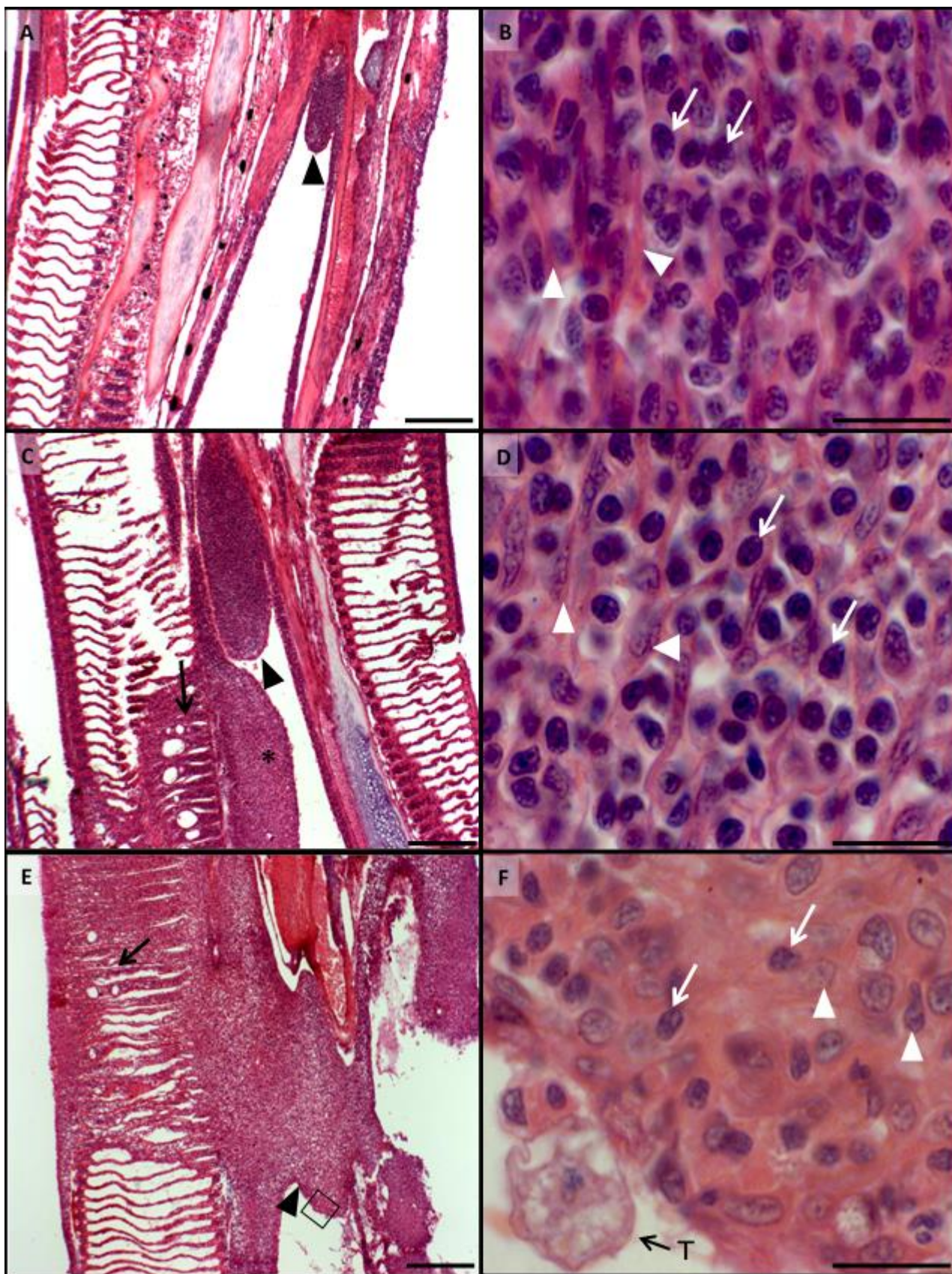


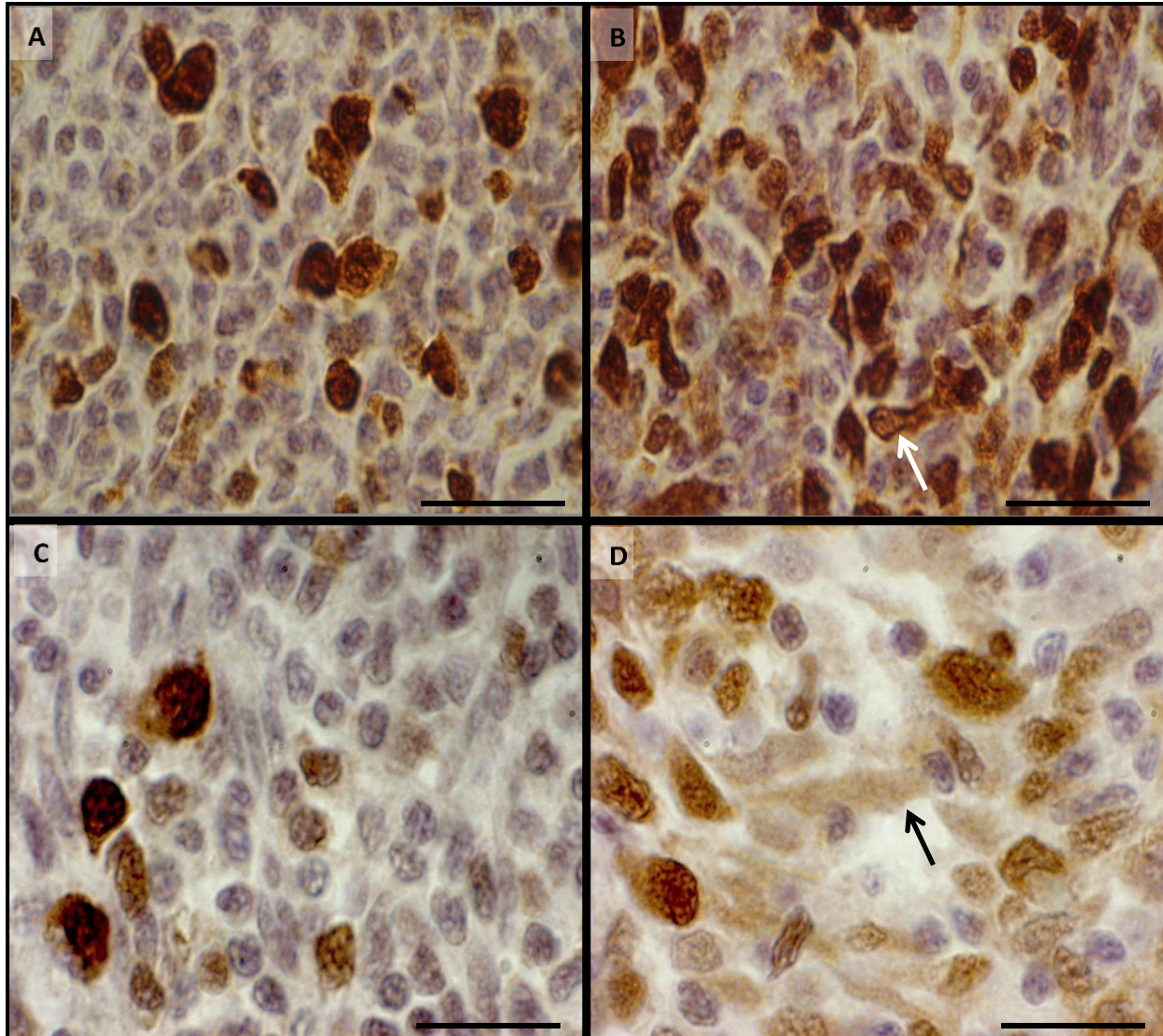
Figure 2. 1. Relationship between the AGD severity (percentage of filaments affected) and days post exposure ( $F = 136.54$ ,  $df = 1, 28$ ,  $P < 0.001$ ).







*Figure 2. 2. Transversal section of a gill filament of a control fish sampled at 14 DPE showing ILT (arrowhead), H&E, Bar = 250µm; B) Detail of A) showing a normal structure, with agglomeration of lymphocytes (arrows) supported by epithelial cells (arrowheads), H&E, Bar = 25µm; C) Transversal section of a gill filament of an AGD affected fish sampled at 14 DPE, showing an enlarged ILT (arrowhead), AGD lesion in the lamellae, presenting hyperplasia and lamellar fusion (arrow), and the extension of the epithelial tissue presenting hyperplasia (asterisk) H&E, Bar = 250µm; D) Detail of C) showing lymphocytes (arrows) and epithelial cells (arrowhead) H&E, Bar = 25µm; E) Transversal section of a gill filament from an AGD affected fish sampled at 14 DPE, showing hyperplasia in the affected lamellae, with fused lamellae (arrow). ILT with an irregular shape (arrowhead). H&E, Bar = 250µm F) Detail of E (marked with a black box in E)), with associated trophozoite (T), presenting cellular changes in the ILT, with a noticeable presence of epithelial cells (arrowheads) and lymphocytes (arrows) H&E, Bar = 25µm.*



*Figure 2. 3. Proliferating cell nuclear antigen (PCNA) staining for detection of cell proliferation, (A) Control fish 14 DPE, with few cells positive for PCNA when compared with (B) AGD affected fish 14 DPE, showing a higher number of proliferating cells, presenting an elongated shape, suggesting to be epithelial cells (arrow) , (C) Control fish 28 DPE presenting few PCNA positive cells when compared with (D), AGD affected fish 28 DPE, showing numerous PCNA positive cells with an elongated shape, suggesting that the positive cells were epithelial cells (arrow) ( Bar = 25 $\mu$ m).*

### *2.3.2 Factors Affecting the ILT*

There was a significant difference in the area of the ILT for the different gill sites ( $F=15.764$ ,  $df=2.216$ ,  $P < 0.001$ ) (data pooled from the different DPE). In uninfected fish the area was

significantly larger in the ventral site of the gill arch than in the dorsal ( $P= 0.002$ ) and medial ( $P< 0.001$ ). In infected fish, the area of the ILT in the medial site was significantly smaller than both dorsal and ventral sites ( $P= 0.033$  and  $P= 0.001$ , respectively) (Figure 2. 4). The interaction between treatment and day post-exposure had a significant effect on the area of the ILT ( $F=6.301$ ,  $df=4,192$ ,  $P< 0.001$ ). The area of the ILT of infected fish at day 28 was significantly larger than the area of the ILT of control fish at the same time point ( $P= 0.001$ ). Furthermore, the area of the ILT in fish affected by AGD was significantly larger 28 DPE when compared with the area of the ILT of fish prior to infection (time 0) ( $P< 0.001$ ), and infected fish at day 3 and 7 ( $P< 0.001$  for both time points) (Figure 2. 5).

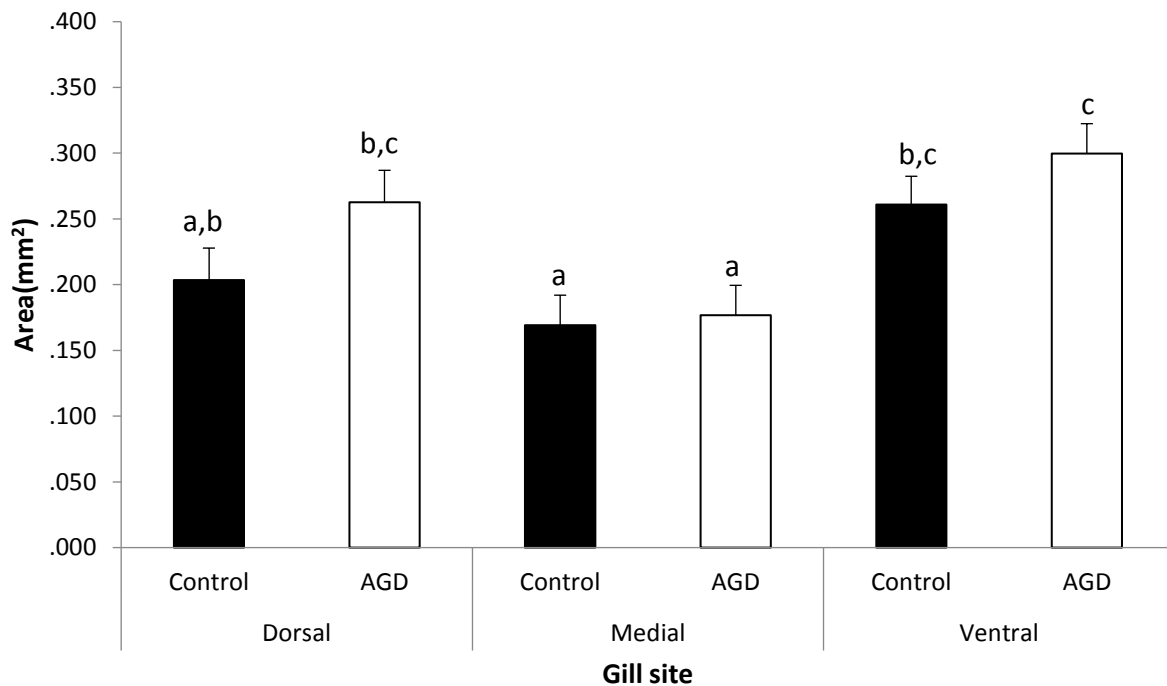


Figure 2. 4. Area of the ILT within different gill sites (area (mm<sup>2</sup>)) for control fish and Infected fish. Values are the pooled mean  $\pm$  SEM of samples collected at all time points for each treatment and gill site.

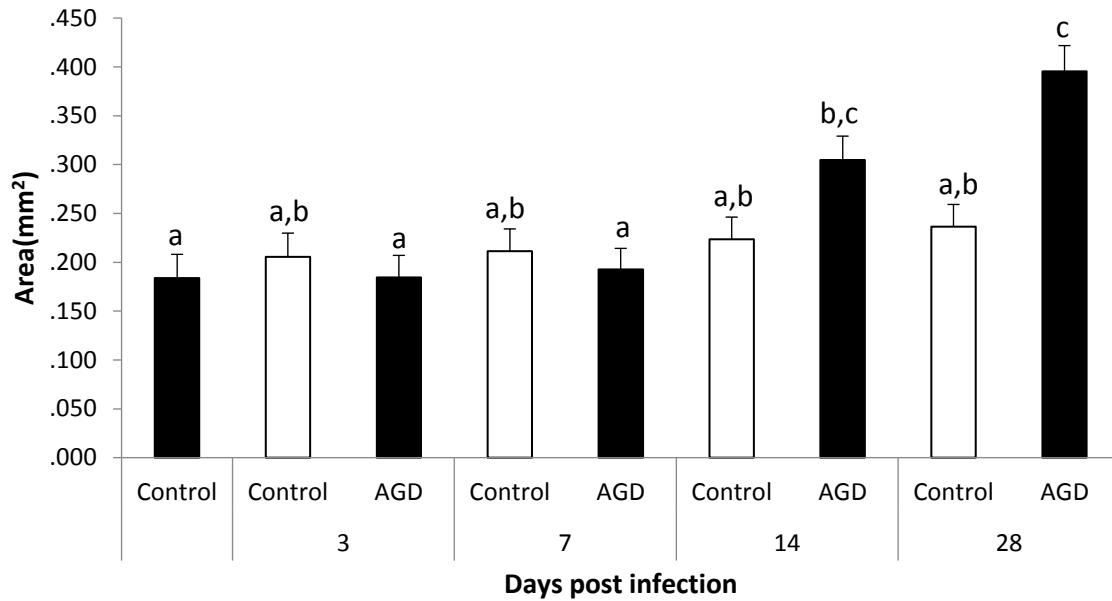


Figure 2. 5. Effect of AGD and time post infection on area of the ILT (area (mm<sup>2</sup>)) at 0, 3, 7, 14 and 28 DPE. Values are mean ± SEM. Different letters indicate values that are significantly different at  $P < 0.05$ .

There was a significant 3-way interaction among day, treatment and gill site on ILT length ( $F = 3.197$ ,  $df = 8, 192$ ,  $P = 0.002$ ). When analysed separately by site there was a significant interaction between day and treatment for the dorsal area of the gill arch ( $F = 3.639$ ,  $df = 9, 64$ ,  $P = 0.001$ ). Post hoc analysis revealed that ILT length was significantly greater 28 DPE in the infected fish than in the control fish at the same time point ( $P = 0.001$ ). Furthermore, ILT length was greater in the infected fish at 28 DPE compared to 3, 7 and 14 DPE ( $P = 0.002$  for all the time points) (Figure 2. 6). Regression analysis showed that AGD severity accounted for 40% of the variation of the area of the ILT ( $F = 18.670$ ,  $df = 1, 28$ ,  $P < 0.001$ ). For the length of the ILT 27% of the variation could be explained by AGD severity ( $F = 10.119$ ,  $df = 1, 28$ ,  $P = 0.004$ ) (Figure 2. 7).

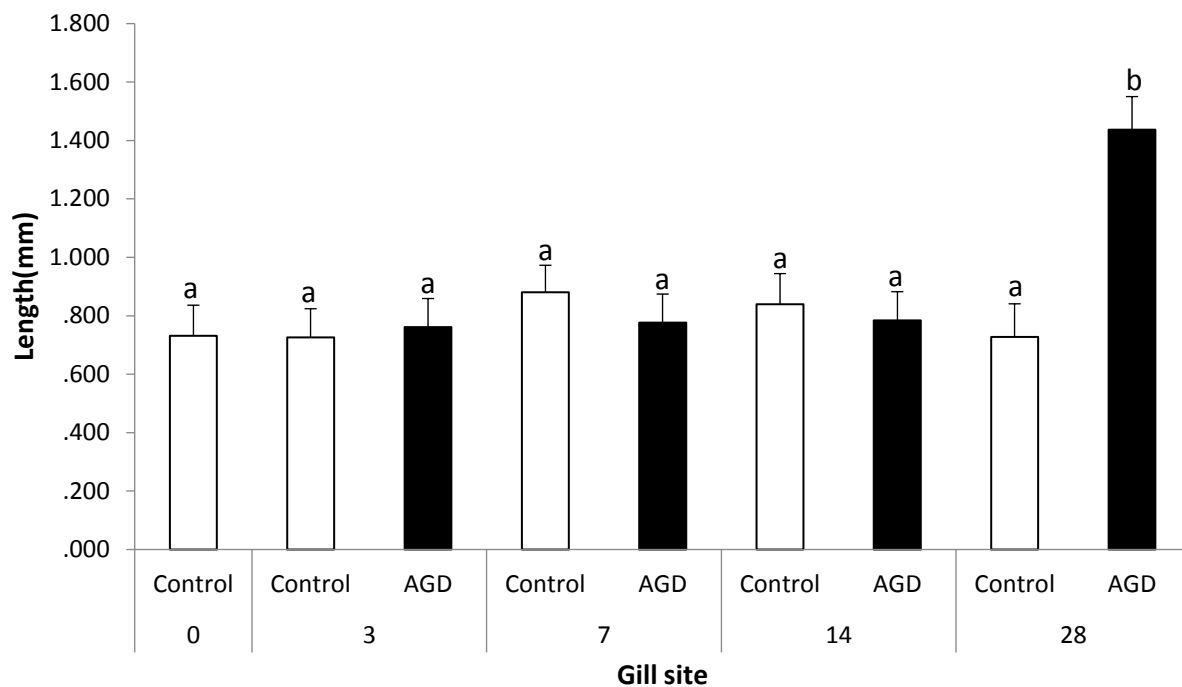


Figure 2. 6. Effect of AGD and time post infection on the length of the ILT ( $\text{length (mm)} / \log (\text{weight}) + 1$ ) in the dorsal site of the gill arch at 0, 3, 7, 14 and 28 DPE. Values are mean  $\pm$  SEM. Different letters indicate values that are significantly different at  $P < 0.05$ .

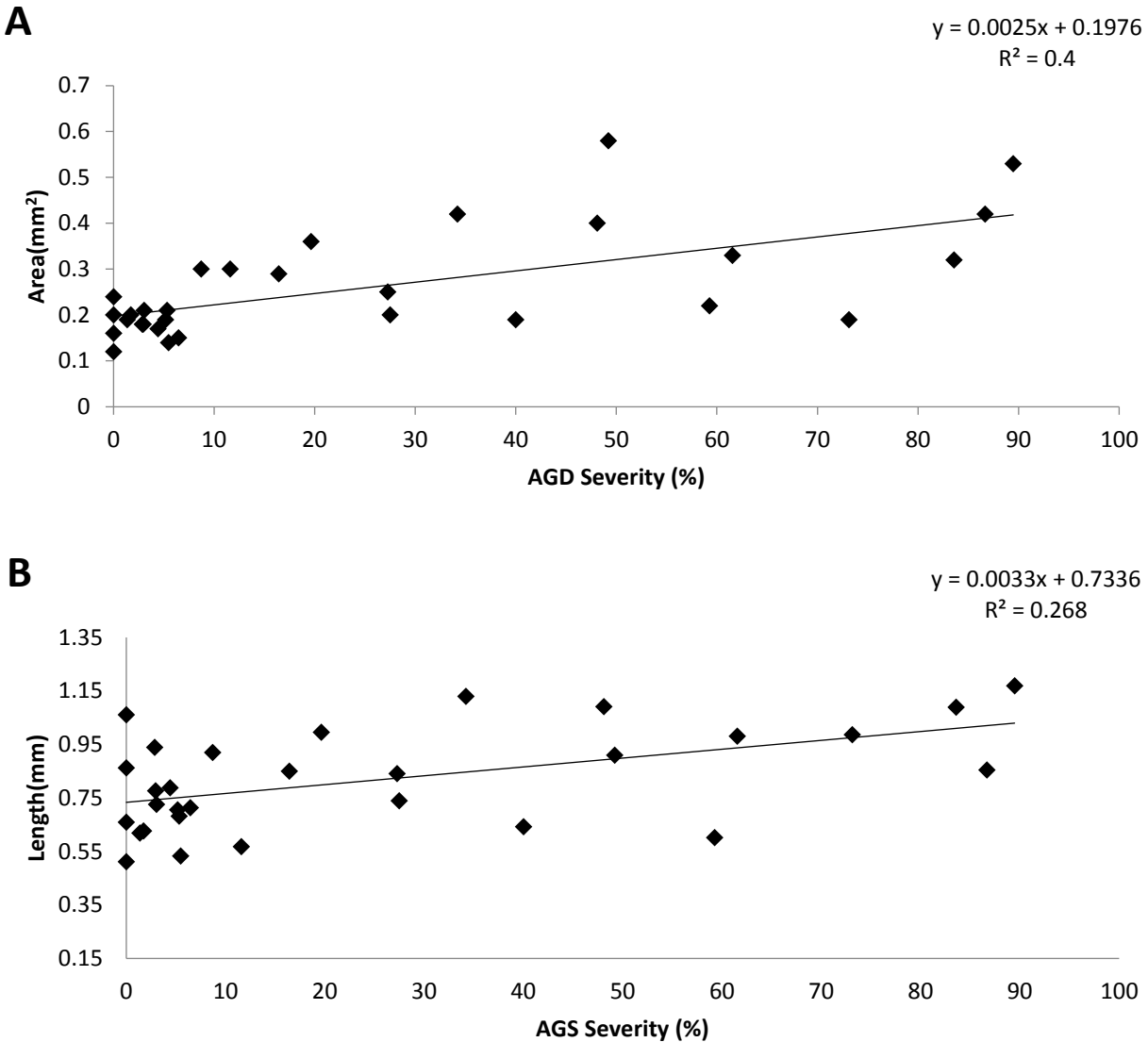


Figure 2. 7. Relationship between the (A) Area of the ILT (area (mm<sup>2</sup>)/log (weight) + 1) ( $F= 19.79$ ,  $df=1,28$ ,  $P< 0.001$ ) and (B) Length of the ILT (length (mm)/log (weight) + 1) and AGD severity expressed as percentage of filaments affected by AGD lesions ( $F=10.337$ ,  $df=1,28$ ,  $P= 0.003$ ).

There was no significant difference in the cell density among the gill sites ( $F=1.980$ ,  $df=2,216$ ,  $P= 0.140$ ). There was a significant interaction between days post-exposure and treatment in the lymphocyte density in the ILT ( $F= 4.618$ ,  $df= 4,192$ ,  $P= 0.001$ ). In fish affected by AGD the density of lymphocytes in the ILT increased significantly 7 DPE when compared with day 0 ( $P= 0.028$ ). At 28 DPE there was a significant decrease in the density of lymphocytes in the ILT of infected fish, when compared with the day 7 ( $P= 0.021$ ). Furthermore, the density of

lymphocytes in the ILT at 28 DPE was significantly lower in infected fish than control fish ( $P=0.032$ ). There was a significant increase in lymphocyte density in the ILT of control fish at 3 DPE. However, the lymphocyte density in this group didn't change significantly over the subsequent time points (Figure 2. 8).

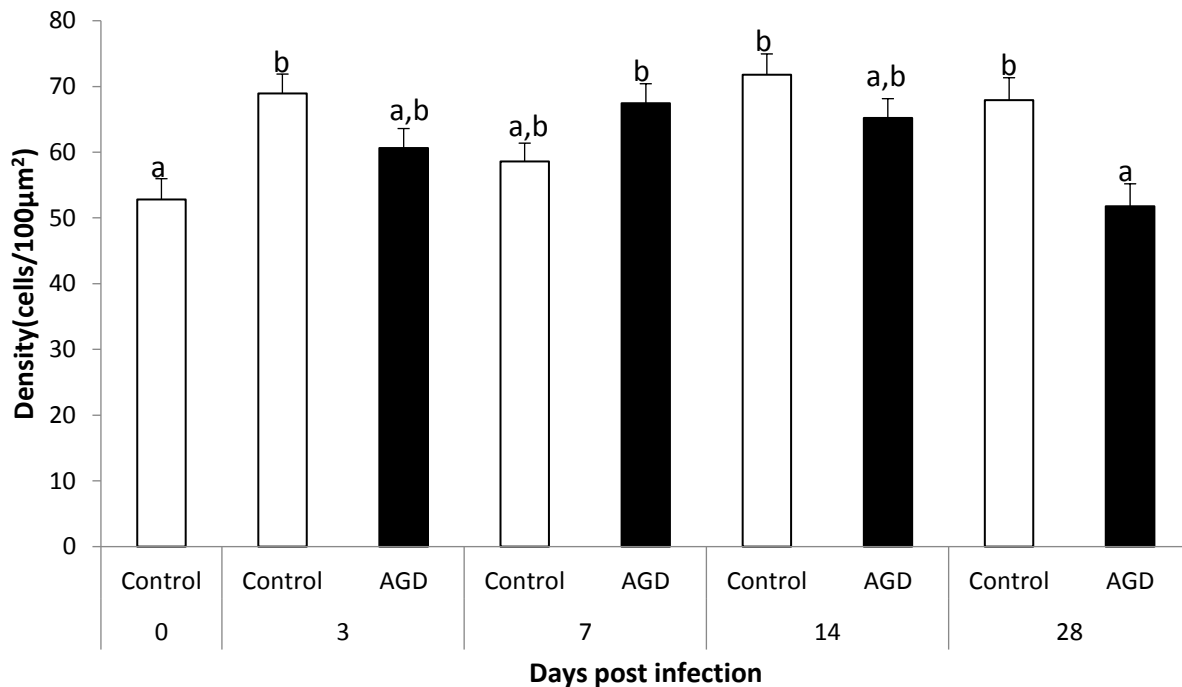


Figure 2. 8 Effect of AGD and time post-exposure on the density of lymphocytes in the ILT of fish at 0, 3, 7, 14 and 28 DPE. Values are mean  $\pm$  SEM. Different letters indicate values that are significantly different at  $P < 0.05$ .

## 2.4 Discussion

*N. perurans* infection caused a remodeling in the ILT of fish affected by AGD. The ILT restructuring was more marked when AGD lesions were present in the immediate vicinity of the ILT and especially obvious in the two individuals where trophozoites were associated with the ILT. In those sections the ILT was enlarged and presenting cellular and morphological changes. The high variability of histological changes observed in the ILT of infected fishes was most likely due to the location of the AGD lesions and the presence of amoebae (Adams and Nowak, 2001). The density of lymphocytes in the control samples was higher at 3 DPE, and a higher variance was found in this group. There was no explanation for this variability and for the increased lymphocyte density after the time 0 DPE.

The lymphocyte density in the ILT of fish affected by AGD was highest at 7 DPE and then decreased. The number of lymphocytes frequently increases in lymphoid organs, especially in the thymus and kidney during an inflammatory response (Secombes and Manning, 1982; Ellis, 1977). The number of lymphocytes in the pronephros of carp experimentally infected with blood fluke (*Sanguinicola inermis*) increased considerably with an increased number of adult flukes at 5 weeks post-exposure and with eggs at 9 weeks post-exposure. At 9 weeks post-exposure, however, the number of lymphocytes decreased significantly (Ellis, 1977). A similar pattern was found in the spleen, pronephros and mesonephros of carp maintained at 20°C and stimulated with an antigen (sheep red blood cells) (Rijkers, 1980). The total number of antibody-producing cells increased, reaching its peak 10 to 15 days post stimulation, and then their numbers declined (Rijkers, 1980).

Despite the lack of specific antibodies to accurately identify the proliferating cells as epithelial cells, the morphology of the cells positive for PCNA suggested that epithelial cells were the main type of proliferating cells, and were more active in AGD affected fish, when compared with control fish. Hyperplasia of the epithelial cells in the ILT of infected fish might be the reason of the lower density of lymphocytes in the ILT. Epithelial hyperplasia is a characteristic of AGD lesions (Adams and Nowak, 2003; Young et al., 2008b), and hypertrophic epithelial cells, with a rounded nucleus comprising scattered clumps of heterochromatin and a distinctive nucleolus were also described (Young et al., 2008b). These changes were observed in the present study, and more evident in the ILT with associated trophozoites. PCNA staining was fainter in the nucleus of hypertrophic epithelial cells. Since PCNA is present in the DNA, when the nucleus is enlarged with finely granular chromatin with irregular distribution, the staining is less dense when compared with the nucleus of normal cells. This can also explain why there appear to be less proliferating cells in affected fish at 28 DFE. This may indicate that the changes observed in the ILT were a typical AGD lesion in a non-respiratory gill surface.

The decrease in the number of lymphocytes in the ILT of AGD affected fish at 28 DPE was possibly caused by migration of lymphocytes from ILT to other sites of the gills, for example AGD lesions. Lower numbers of certain cells in the lymphoid organs have been suggested to be related to the migration of these cells out of these organs and into sites of inflammation (Richards et al., 1994). Increased numbers of immune cells have been found at the site of AGD



lesions (Adams and Nowak, 2003; Adams et al., 2004; Gross et al., 2005). It has been suggested that the leucocytes present in the sites of AGD lesions could have been recruited from other leucocyte populations such as peripheral blood, or lymphoid organs such as head kidney or spleen (Gross et al., 2005). A depletion of D8 $\alpha$ + cells was described in the ILT of rainbow trout infected with ich (*Ichthyophthirius multifiliis*) (Olsen and Kania, 2011). Due to the presence of the CD8+ material inside vacuoles in Ich trophonts, it was suggested that depletion was due to ingestion of those by Ich (Olsen and Kania, 2011). However, in the present study it is more likely that due to the location of the ILT, the population of leucocytes has been recruited to the sites of infection, thus their depletion. A reduction of the size of the ILT in Atlantic salmon infected with ISAV was suggested to be caused by the migration of leucocytes (Austbo et al., 2014; Hetland et al., 2010). Thus, the ILT may function as a reservoir of lymphocytes (Austbo et al., 2014). However, at the moment there is not enough evidence to validate this hypothesis.

Suppression of leucocyte proliferation in the spleen of roach (*Rutilus rutilus*, L.) in response to extracts from *Lingula intestinalis* in an in vitro study was likely due to its cytotoxicity to spleen lymphocytes (Taylor and Hoole, 1994). A cytopathic effect of *Neoparamoeba pemaquidensis* in the gill epithelium of Atlantic salmon has been suggested (Butler and Nowak, 2004; Lovi and becker, 2007). A protease-like exotoxin released by *N. perurans* has been proposed to stimulate the necrosis of epithelial cells seen ultrastructurally in affected Atlantic salmon (Nowak and Morrison, 2004). There was a depletion of lymphocytes in the ILT of AGD affected fish 28 DPE, however the size of the ILT increased with the increased time of infection. This may be due to the proliferation of epithelial cells, which is a characteristic of AGD lesions, with a resultant increasing ratio of stromal cells to lymphocytes.

The ILT of infected fish enlarged significantly, reaching its peak at 28 DPE. Measurements of the ILT of infected fish sampled 28 DPE were more laborious, due to very irregular shape of the ILT observed at this time point, making the use of image analysis difficult. The measuring technique used was analogous to the other time points for consistency, tracing the ILT from the basal area until the top, and avoiding including the epithelium of the gill filament (in the lateral site of the ILT). When it was impossible to perform image analysis in one of the sections new recuts were made, and if it was still impossible to analyse the sections, the individual was excluded from the analysis. Changes in the length of the ILT were only significant in the dorsal

site of the gill arch. According to (Adams and Nowak, 2001), the number of AGD lesions and pathological severity was higher in the dorsal site of the gill arch than the ventral site. This could be an explanation for the significant increase in the length of the ILT in the dorsal site 28 DPE. *N. perurans* was not always associated with changes in the ILT. Therefore, it is unlikely that the increased size of the ILT was just attributed to the presence of trophozoites. Later stages of AGD lesion comprise a substantial epithelial rearrangement, and these regions are rarely colonized by trophozoites (Adams and Nowak, 2003). However, it needs to be considered that trophozoites might be present in the ILT, but due to deepness or angle of section it might not be shown in the histological sections. Furthermore, the proximity of AGD lesions, even when not detected in the histological sections analysed, may have had contributed to the changes observed in the infected fish and could be associated with the progression of the lesion from the lamellae to the interbranchial septum. AGD lesions progress along the filaments and this was attributed to migration of proliferating amoeba (Adams and Nowak, 2003). This might have contributed to the morphological changes in the ILT. This elongation may occur along the filament, but also towards the interbranchial septum, thus affecting the ILT.

Parasitism can have a significant effect on the cellular composition of lymphoid organs (Richards et al., 1994). Enlargement of the spleen (splenomegaly) was reported in parasitic infections, such as a diplomonad flagellate, *Spironucleus barkhanus* (Hexamitidae) in Atlantic salmon (Guo and Woo, 2004) and a haematozoic endoparasite, *Trypanoplasma borreli* in common carp (*Cyprinus carpio* L.) (Bunnajirakul. et al., 2000). Splenomegaly was attributed either to proliferation of lymphocytes and granulocytes in carp (Secombes and Manning, 1982; Secombes et al., 1982), or retention of erythrocytes in rainbow trout and Atlantic salmon (Bruno and Munro, 1986). An increased number of lymphocytes was reported in lymphoid organs of carp and rainbow trout, especially thymus and spleen during the inflammatory response (Richards et al., 1994; Secombes et al., 1982; Tatner and Tatner, 1985). In this study, the increased area of the ILT in AGD affected fish did not appear to be caused by lymphocyte proliferation. Although there was proliferation of lymphocytes at day 7 post-exposure, lymphocyte density was lower in the infected fish measured on day 28. Studies in mammalian lymph nodes response to pathogens have shown that the reticular cell network expands by proliferation and contributes to enlargement to allow greater accommodation of more lymphocytes (Yang et al., 2014). In the present study, an increased number of lymphocytes was

not detected in the later stages of infection, so it is unlikely that they contributed to the enlargement of the ILT. This could be caused by hyperplasia of epithelial cells, which is characteristic of AGD lesions (Bunnajirakul, et al., 2000; Morrison et al., 2004). It is possible that the ILT responds similarly to the epithelium in the filaments affected by AGD. Previous research on fish thymus suggests that epithelial cells have an important role in the maturation and acquisition of thymocytes, by producing humoral factors that induce the development and expression of thymic hormones (Yang et al., 2014). Furthermore, a secretory role of epithelial cells has been suggested (Castillo et al., 1990). Tissues reorganization has been studied in mammals, and the fibroblastic reticular network has been reported to have an important function not just to support the structure and compartmentalization of secondary lymphoid organs, but also in regulation of cell distribution in the lymph nodes, by signaling through tumor necrosis factor-lymphotoxin (Drayton et al., 2006). Moreover, it has been shown that reticular cells express certain chemokines that provide guidance to T cells, which preferentially move along the network filaments (Graw, and Regoes, 2012; Bajenoff et al., 2006; Bajenoff et al., 2008, Nolte et al., 2003). In the ILT, the hyperplasia of the epithelial cells may be linked to lymphocyte migration to the lesion. However, further research with specific cell markers should be conducted, to highlight the function of these cells in the immune response to AGD.

## 2.5 Conclusion

AGD induced a morphological and cellular response in the ILT, resulting in remodeling of the ILT. Epithelial hyperplasia was the most likely factor contributing to the ILT enlargement. Further work in cell characterization will be useful for further clarification of ILT function during AGD. The results of this research showed for the first time the response of the ILT to a marine parasite. Furthermore, this study contributes to future elucidation of ILT function in AGD.

## Acknowledgements

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# Chapter Three

## The Morphometry and Cellularity of Interbranchial Lymphoid Tissue in Atlantic Salmon Following Experimental Re-Infection with *N. perurans*

### 3.1 Introduction

Interbranchial lymphoid tissue (ILT) is located along the interbranchial septum (proximal ILT) and extends throughout the trailing edge of the filament (distal ILT) (Dalum et al., 2015). The ILT is mostly composed of T-cells embedded in a meshwork of epithelial cells (Dalum et al., 2015; Haugarvoll et al., 2008; Koppang et al., 2010). The T-cell receptor (TCR) present upon the surface of T-cells specifically recognises externally presented antigens in conjunction with the CD3 receptor complex (Nam et al., 2003). This molecule has a cytoplasmic tail that is the signalling region, which is comprised of different chains: CD3 $\gamma\delta$ , CD3 $\zeta$  and CD3 $\epsilon$ . Each TCR complex has two CD3 $\epsilon$  molecules, which makes it an ample target for antibody binding and detection (Boardman et al., 2012). CD3+ cells can be divided into two main types: CD4+ cells (T-helper cells) and CD8+ (cytotoxic T-cells). In the ILT, a significant population of T-helper cells (CD4+ cells) has been described using molecular analyses (Aas et al., 2014; Austbo et al., 2014 Dalum et al., 2015). Additionally, a minor population of cytotoxic T-cells (CD8+) was revealed using immunohistochemistry (Aas et al., 2014; Austbo et al., 2014 Dalum et al., 2015).

Amoebic gill disease (AGD) is a proliferative gill condition caused by a parasitic marine amoeba, *Neoparamoeba perurans*. AGD is now a significant health problem for marine aquaculture worldwide (Young, Dykova et al. 2008). The pathological onset of AGD is concomitant with epithelial desquamation and oedema in the area where the trophozoite is attached (Adams and Nowak, 2004). Hyperplasia of epithelial cells lead to lamellar fusion

leading to gross-observable lesions, which are characteristic of this disease (Adams and Nowak, 2003; 2004). A strong cellular immune response is observed, with a high number of neutrophils and macrophages in the hyperplastic lesions and occasionally infiltrate interlamellar vesicles (Adams and Nowak, 2001; 2004). Furthermore, a reduction of chloride cells has been observed in lesion areas (Adams and Nowak, 2003; Nowak and Munday, 1994). The infiltration of T-cells, in particular cytotoxic T-cells and MHC class II was also reported in AGD lesions (Morrison, Cooper et al. 2006; Pennacchi et al., 2014). Several studies have found that pathological changes observed in the gills are complemented by inflammatory immune responses (Koppang et al., 2015). Pennacchi et al. (2014) revealed an up-regulation of pro-inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) in response to *N. perurans*. Furthermore, the same study revealed that there was an up-regulation of genes related to adaptive cell-mediated immune responses (MHC class I and class II, TCR- $\alpha$ , CD8-  $\alpha$  and CD4), as well as humoral immune response with an up-regulation of IgM and IgT (Pennacchi et al. 2014).

Previous studies have focussed upon cellular changes in the respiratory regions of the gills. In these regions AGD causes a localised inflammatory response where lymphocytes, neutrophils and macrophages have been shown to infiltrate hyperplastic lesions (Munday et al., 1990; Adams and Nowak, 2001; 2003). However, ILT and its likely contribution to immune cell trafficking in the gills of salmonids is only just emerging as a target for investigation. During a naïve experimental infection of Atlantic salmon with *N. perurans* the, pILT area was larger when disease was more severe during the later stages of infection possibly due to hyperplasia of epithelial cells (Norte dos Santos et al., 2014). Concurrently, there was a reduction in lymphocyte density in the pILT of which was more apparent when the pILT was adjacent to lesions (Norte dos Santos et al., 2014). It was suggested that lymphocytes could be migrating from the pILT to interlamellar lesions (Norte dos Santos et al., 2014). A similar conclusion was reached when increased transcriptional expression of TCR mRNA was correlated with an increase of CD8 mRNA in AGD lesions following an experimental naïve infection in the same species (Pennacchi et al., 2014). Several studies have shown that the susceptibility of Atlantic salmon to amoebic gill disease differs from individual to individual, and two main factors may contribute to this difference: the uneven spread of the disease through the population, and individuals from the same population may have different susceptibility levels (Wynne et al., 2007, Taylor et al., 2007; Taylor et al., 2009). Both laboratory controlled and field challenges

have found genetic variation in gill scores, as measured by heritabilities and genetic differences for both gross gill pathology and survival (Kube et al., 2012; Taylor et al., 2007; Taylor et al., 2009). The genetic resistance to AGD manifests in two distinct traits: resistance of naïve fish at first infection and a form of adaptive resistance that is expressed after reinfection (Kube et al., 2012). Atlantic salmon experimentally infected with *N. perurans* have shown an increase of MHC class II cells in the gill epithelium, suggesting that immune cell migration and antigen presentation was occurring (Morrison et al., 2006). Studies on the expression of immune genes in the gills of Atlantic salmon revealed that there was little difference in the expression of cell markers (TCR- $\alpha$  chain, CD8, CD4, MHC-II $\alpha$ , MHC-I) and immunoglobulins (IgM and IgT) between unexposed, naïve fish and those repeatedly exposed to *N. perurans* (Pennacchi et al., 2016). A previous study found significant differences in the expression of immune genes between uninfected and non-infected fish, and also between areas with lesions vs non-lesion (Pennacchi et al., 2014). However, little difference was found in the gene expression of immune related genes in the gills of AGD affected fish between singly and repeatedly exposed to *N. perurans*. The differences in gene expression between the lesions and areas where lesions are not present, may be indicative that the ILT may influence the localised immune response. However, no previous study has compared the ILT of fish that were singly and repeatedly exposed to *N. perurans*.

A strong cellular response has been documented in the gills of Atlantic salmon during AGD. Differences in susceptibility have been found among individuals, being suggested that genetics may play a role in the predisposition to AGD. It is likely that the ILT is involved in AGD response and differences in its cellularity and morphology may be present between naïve fish and those repeatedly exposed to *N. perurans*. The present study aimed to compare the histopathology (morphometry and abundance of T-cells) in the ILT of fish that were singly and repeatedly exposed to *N. perurans*. Transversal sections were cut and stained with H&E and the number of T-cells was identified via the CD3 $\epsilon$  marker developed by Koppang et al. (2010). The size of the proximal ILT was measured, as well as the number of CD3+ cells, and results compared between singly and repeatedly exposed fish. Histopathology assessment from proximal and distal ILT was performed and compared between singly and repeatedly exposed fish.

## 3.2 Materials and Methods

### 3.2.2 *Ethical Approval*

This study fulfilled the guidelines of the Australian code of practice for the care and use of animals for the scientific purposes was approved by University of Tasmania's Animal Ethics Committee (Reference A0012237).

### 3.2.3 *Fish History Prior to Infection with *N. perurans**

Commercially reared Atlantic salmon (n= 307, mean weight 55g) were transferred from a commercial hatchery and habituated to two twin tank freshwater recirculating systems (4000 l/system) for one month. Fish were fed a 3mm sinking pellet (Skretting Spectra & Spirit Supreme) at 2% of body weight per day. The water quality was maintained at salinity 0‰, > 85% dissolved oxygen,  $\leq 1 \text{ mg l}^{-1}$  TA-N,  $< 2 \text{ mg l}^{-1}$  NO<sub>2</sub>,  $\leq 40 \text{ mg l}^{-1}$  NO<sub>3</sub>, pH 6.8-7.2 and temperature of 15.5°C.

After habituation to the system, the fish were lightly sedated ( $5 \mu\text{l l}^{-1}$  clove oil) and transferred in groups of ten to a 100 l tub filled with water (from the same tank the fish were in) with clove oil ( $30 \mu\text{l l}^{-1}$ ). Once fish were non-responsive to peduncle squeeze, a small incision was made with a sterilized scalpel blade in the anterior area of the base of the right pelvic fin. A sterile passive integrated transponder (PIT) tag was inserted into the intraperitoneal cavity. The incision was dressed with a sealant mixed with antibiotic (2% w/w Tricon). Pit Tag marked fish were placed back into their original tanks, for acclimation to saltwater (temperature = 15.5°C, salinity 0‰, pH 6.8-7.2, TA-N  $\leq 1.0 \text{ mg.l}^{-1}$ , NO<sub>2</sub>  $< 2 \text{ mg.l}^{-1}$ , NO<sub>3</sub>  $\leq 40 \text{ mg.l}^{-1}$  and DO<sub>sat</sub> >85% ). After the acclimation phase (126 days), fish were sedated and transferred temporarily to a single tank with oxygenation. They were then randomly relocated to either a twin tank (2000 l system) (Atlantic salmon n= 50; Rainbow trout n =40 stock density:  $9.3 \text{ kg m}^{-3}$ ) and remained unexposed to *N. perurans* for the duration of the trial or returned to the original system (twin tanks 4000 l system) (Atlantic salmon n= 181 Rainbow trout n =151; stock density:  $16.9 \text{ kg m}^{-3}$ ) and repeatedly exposed to *N. perurans*. Atlantic salmon were held in tanks with equivocally sized rainbow trout until the first treatment for AGD. The stocking densities presented are inclusive of rainbow trout held in the systems. The fish were PIT-tagged for the confirmation that the fish repeatedly

exposed to *N. perurans* selected for this study presented lesions throughout the experiment. Rainbow trout were added to the experiment to serve as a comparison with Atlantic salmon. However, due to marine acclimation difficulties, they were withdrawn from the experiment.

### *3.3.3 Repeated Exposure Regime (N. perurans)*

For each exposure, trophozoites were isolated from AGD-affected fish as described by Morrison et al., 2004. The timeline for the experiment is shown in Figure 3.1. Gill assessments were made to acquire data for a parallel experiment and to ensure that all the fish analysed were affected by AGD in all gill checks (Pennacchi et al., 2016). The gill check was done to check the progress of the infection, and to evaluate the need for fresh water bath.

### *3.3.4 First Exposure to N. perurans*

After acclimation, fish in the 4000 l system were exposed to *N. perurans* (194 trophozoites l<sup>-1</sup>) (day 0) under static conditions for 6 hours. Mechanical filtration and UV disinfection was stopped for 24 hours, following re-establishment of water flow. At three weeks post-exposure, a subsample of fish (n= 10) was taken from the infection tanks by dip-netting, anaesthetized with clove oil (30µl l<sup>-1</sup>) and inspected for gross gill lesions to confirm the clinical progression of the infection to a diseased state. One week after the gill check a 3 hour freshwater bath was applied for all fish (infected and controls) (see below for detail regarding gill checks and bathing).

### *3.3.5 Second Exposure to N. perurans*

The second exposure to *N. perurans* occurred immediately after the recovery from freshwater bathing (24 hours). The concentration of *N. perurans* trophozoites was 158 trophozoites l<sup>-1</sup>. Mechanical filtration and UV disinfection was stopped for 24 hours, but water circulation was maintained. Seventeen days post second exposure the gills of all the fish were checked for gross signs of disease. Then, the fish were bathed in freshwater, and nine days later another freshwater bath was performed.



### 3.3.6 Third Exposure to *N. perurans*

The third exposure to *N. perurans* occurred after fish recovered from previous freshwater bath (48 hours). The concentration of *N. perurans* was 1000 trophozoites l<sup>-1</sup> and the conditions were the same for the previous exposures. Three weeks post exposure all the fish were checked for gross gill pathology. After the gill check the fish were bathed in freshwater, and were then returned to the experimental tanks in which the water was not changed, assuming that amoebae remaining within the system would disseminate on restocking. Twenty fish from the unexposed but identically managed cohort were added to the infection system in order to provide a naïve (single exposure) group for comparison.

### 3.3.7 Final Exposure to *N. perurans*

Approximately one month following the final freshwater bath an additional 475 *N. perurans* trophozoites l<sup>-1</sup> were added to the infection system. At 4 weeks post exposure the final sampling was performed.

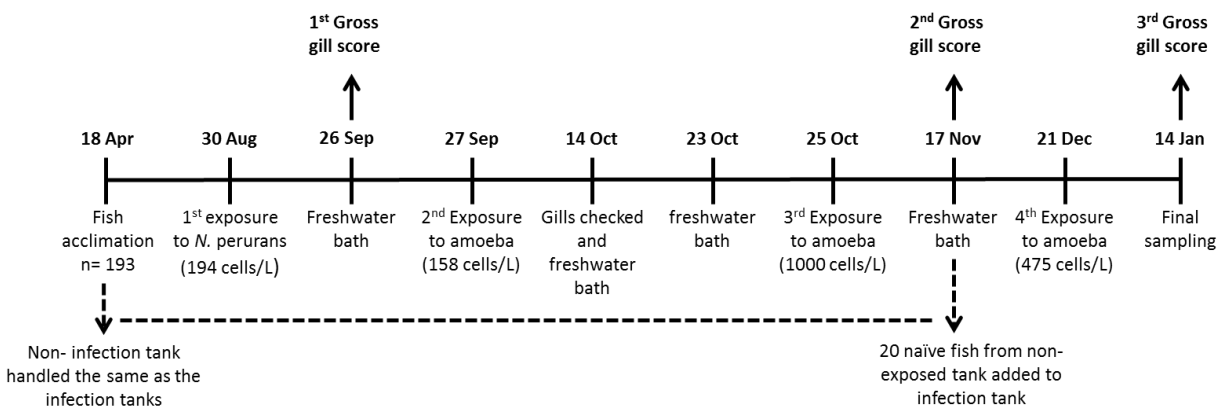


Figure 3. 1. Time-line of experiment showing time between exposure to *N. perurans*, gill scoring and freshwater baths. Gill assessments were not used in the present study.

### 3.3.8 Gross Checks and Freshwater Treatments

Gill checks were made on subsamples (n= 10) of exposed fish to evaluate the clinical progression of AGD during each infection round and determine the need for treatment. These checks were performed approximately 2 weeks post-exposure to *N. perurans*. Immediately prior to treatment, groups of approximately ten fish were transferred by dip-net to a 100 l tank

containing 40 l of water and anaesthetic (30  $\mu\text{l l}^{-1}$  clove oil) until they were unresponsive to touch. The fish were scanned for the PIT tag code, the gills were inspected for AGD lesions. All fish were handled in this manner.

Following gross gill assessment, the fish were bathed for 3 hours in freshwater (15-16°C, pH 7.0, hardness < 20 mg  $\text{l}^{-1}$ ,  $\text{CaCO}_3$ ,) in a 1000L tank with sedation (clove oil 1.5 $\mu\text{l l}^{-1}$ ).

Simultaneously, the experimental tanks were drained, cleaned and filled with seawater adjusted to the correct temperature and salinity, chlorinated with sodium hypochlorite 5 mg  $\text{l}^{-1}$  for two hours and then de-chlorinated with sodium thiosulphate. At the completion of the freshwater bath the concentration of clove oil was increased to 10  $\mu\text{l l}^{-1}$  to facilitate transfer of fish back to the experimental tanks. The handling and treatment of the fish (for gill assessments and freshwater bathing) were the same for exposed and unexposed fish at all stages.

### *3.3.9 Final Sampling*

Each tank level was reduced by 50 % and all fish were removed by dip net (n= 10 per collection) and transferred to a 100 l tub containing seawater from the same tank and clove oil (30  $\mu\text{l l}^{-1}$ ).

Once the fish were unresponsive the weight and length were recorded (mean weight at final sampling = 391,77  $\pm$  112 g). The gills were excised, rinsed in 0.2  $\mu\text{m}$  filtered seawater and then placed into 10 % neutral buffered formalin. Fixed gills were kept in 4°C for 24 hours and remained in formalin until processing at room temperature. Gross gill scoring was used to select fish that represented all spectrum of disease.

### *3.3.10 Histology*

For fish unexposed to *N. perurans* (n=5), three transversal sections of the second left gill arch were excised from the holobranch and placed in a histological cassette. The transversal excisions were collected from the distal, medial and dorsal gill locations. For AGD affected fish (n= 26), For AGD affected fish, filaments were excised under a dissecting microscope. One section comprised filaments with no gross pathology (No Lesion), and two sections from filaments where gross pathology was observed (Lesion). No measurements of the location of the lesion in relation to the ILT were made. Samples were dehydrated and embedded in paraffin according to standard procedures. 5  $\mu\text{m}$  sections were cut and stained with hematoxylin and eosin (H&E), and

4 µm sections were cut to perform immunohistochemistry. An additional gill arch was collected from each fish and similarly processed for assessment of AGD severity.

### *3.3.11 Immunohistochemistry*

Sections (4 µm) were mounted on glass slides (Superfrost®, Menzel, Braunschweig, Germany), incubated (37°C – 20 hours and 58°C – 40 min) and then de-waxed and rehydrated. Sections were then autoclaved in 0.01 M citrate buffer, pH 6.0 at 120°C for 10 min followed by treatment with phenylhydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37°C and rinsed three times in phosphate-buffered saline (PBS). Nonspecific binding was prevented by adding goat normal serum diluted (1:50) in 5% bovine serum albumin (BSA) in PBS. Incubation with the primary antibody, a monoclonal antibody recognizing trout CD3ε (dilution 1:400) (Boardman et al., 2012), in tris-buffered saline (TBS) with 1% BSA was done for 30 min at room temperature, then rinsed three times in TBS and incubated with a secondary antibody (EnVision® System kit; Dako, Glostrup, Denmark) for 30 min. Slides were washed three times in TBS, and incubation with diaminobenzidine (DAB) for 7 min (EnVision® System kit). Sections were washed with distilled water and counterstained with Mayer's hematoxylin for 2 minutes then mounted with polyvinyl alcohol media pH 8.0. Negative controls were incubated with 1% BSA instead of the primary antibody.

### *3.3.12 Histology and Image Analysis*

Sections comprising filaments presenting lesions were histopathologically assessed, including cell quantification and area measurement, by using both H&E and IHC sections. An Olympus BH-2-RFCA (Japan) compound light microscope was used for microscopical assessment. For cell quantification, a single image (100X objective) was captured from the apical and basal region of the proximal ILT (pILT) per section examined (area= 1 mm<sup>2</sup>). The number of cells was quantified manually with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) using the plugin “Cell counter and marking” (Kurt De Vos, University of Sheffield, Academic Neurology <http://imagej.nih.gov/ij/plugins/cell-counter.html>). Cell density was calculated as the number of cells per 1 mm<sup>2</sup>. Cells quantified were CD3<sup>+</sup> (DAB<sup>+</sup> - brown), CD3<sup>-</sup> (counterstain – blue), and ambiguous cells, in which it was unclear if the cell was DAB<sup>+</sup>, to calculate the total number of cells. The area of the proximal ILT was measured using the

program Image Processing and Analysis in Java (ImageJ) as per Norte dos Santos et al. (2014) as follows. A scale was set according to the picture magnification (100X), the polygon tool was used to delineate the proximal ILT, measuring the area of the subsection obtained. The area of the ILT was measured in mm<sup>2</sup>. Severity of AGD was quantified as percent of gill filaments with an AGD lesion according to Adams and Nowak 2003. For this study, only the proximal ILT was used for the quantitative analysis. Inconsistencies in the orientation of dILT sections resulted in few suitable replicates for quantitative analysis. Both the pILT and dILT were used for the histopathological assessment.

### *3.3.13 Data Analysis*

After a pre-assessment of the quality of the sections stained using immunohistochemistry, the number of fish assessed was eight naïve (total of 16 sections), 18 re-exposed (total of 47 sections) and five unexposed (total of 11 sections) (Fig, 3.2). Cell quantification and area measurement (dependent variables) means for density (CD3<sup>+</sup>, CD3<sup>-</sup>, and total number of cells) and area (independent variables) were compared for fish exposed to *N. perurans* using a two-way ANOVA using exposure (single or repeated) and lesion presence as fixed factors.

Unexposed fish data was used as reference (shown in figures) but was not included in the ANOVA analyses. A Pearson's bivariate test was used for correlation analyses for % AGD severity [(Number of AGD lesions/Number of filaments) \* 100], pILT area, number of CD3<sup>+</sup> cells, number of CD3<sup>-</sup> cells and number of total cells. Assumption of normality was checked with the Shapiro-Wilk test and the homogeneity of data was analyzed using the Levene's test. A *p* value of < 0.05 was adopted for the rejection of the null hypothesis but when the assumptions failed, the significance level was lowered to 0.01 (Underwood, 1981).

## **3.4 Results**

### *3.4.1 Histopathological Assessment*

The pILT of a non-exposed fish presented as a meshwork of epithelial cells surrounding lymphocytes. The apical area presented lymphocytes embedded in the epithelium (Figure 3.2, A). The dILT of a non-exposed fish presented a similar structure, with mucous cells apically present, a meshwork of epithelial cells surrounding lymphocytes and a basement membrane

(Figure 3.2, B). Histopathological assessment revealed that AGD caused cellular and morphological changes in both proximal (pILT) and distal (dILT) ILT. The cellular and morphometric changes observed in the pILT and dILT were highly variable not only among fish from the same exposure groups, but also among sections of the same gill arch. Histopathological changes were more evident in sections adjacent to lesions, both for the pILT and dILT.

The major differences observed between the singly and repeatedly exposed fish both in the dILT and pILT was that the singly exposed fish often presented a large degree of restructuring than the repeatedly exposed fish. When in filaments that presented lesions, the pILT of singly exposed fish presented a high number of hypertrophic epithelial cells, with stippled chromatin, apoptotic cells and oedema (Figure 3.43). In the pILT and dILT of repeatedly exposed fish it was common to observe a higher number of cells (including CD3<sup>+</sup> cells) (Figure 3.4, A and B). One common observation for the pILT adjacent to filaments that presented lesions was a denser layer of epithelial cells apically often devoid of mucous cells (Figure 3.44, A). Scattered small eosinophilic mononuclear cells (EMNC) were observed in the proximal ILT (Figure 3.44, A, B).

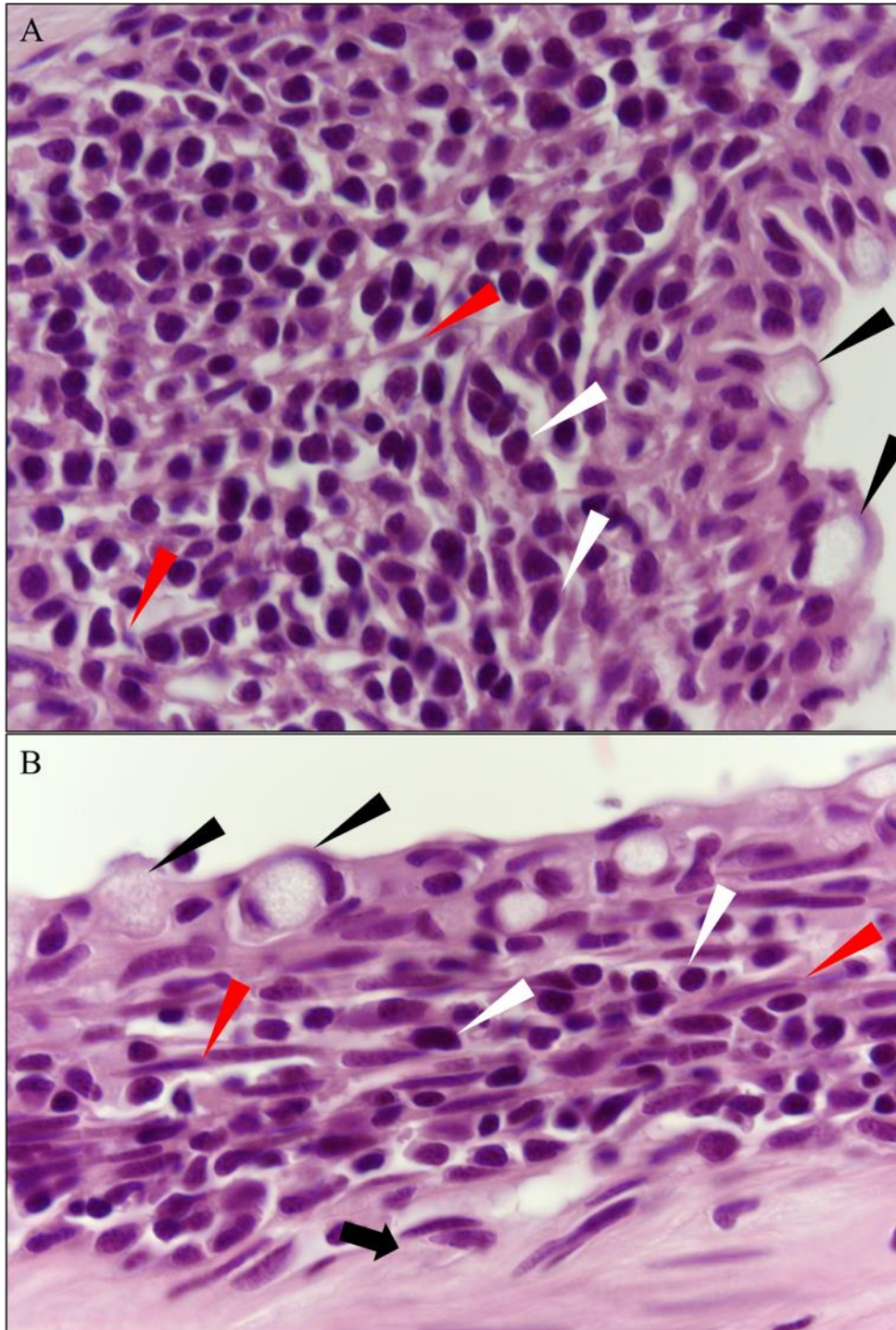
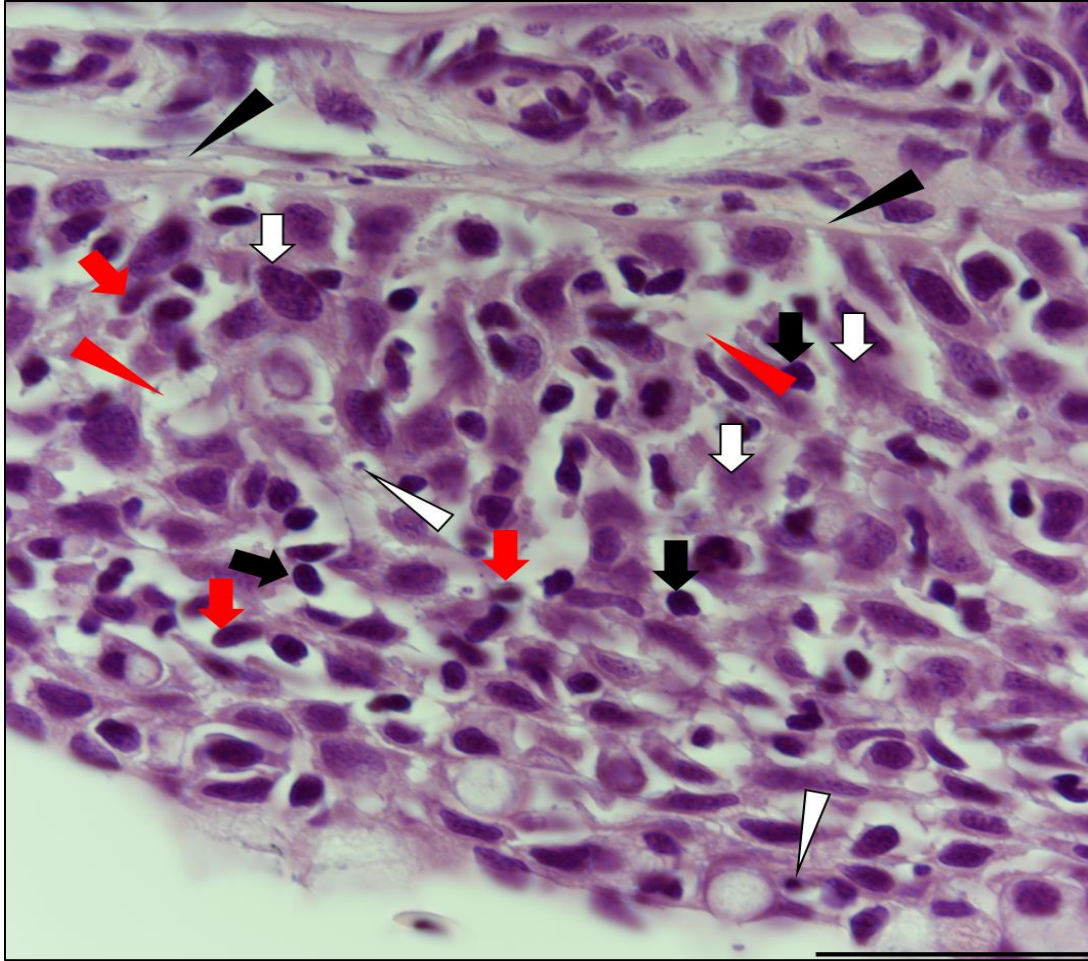
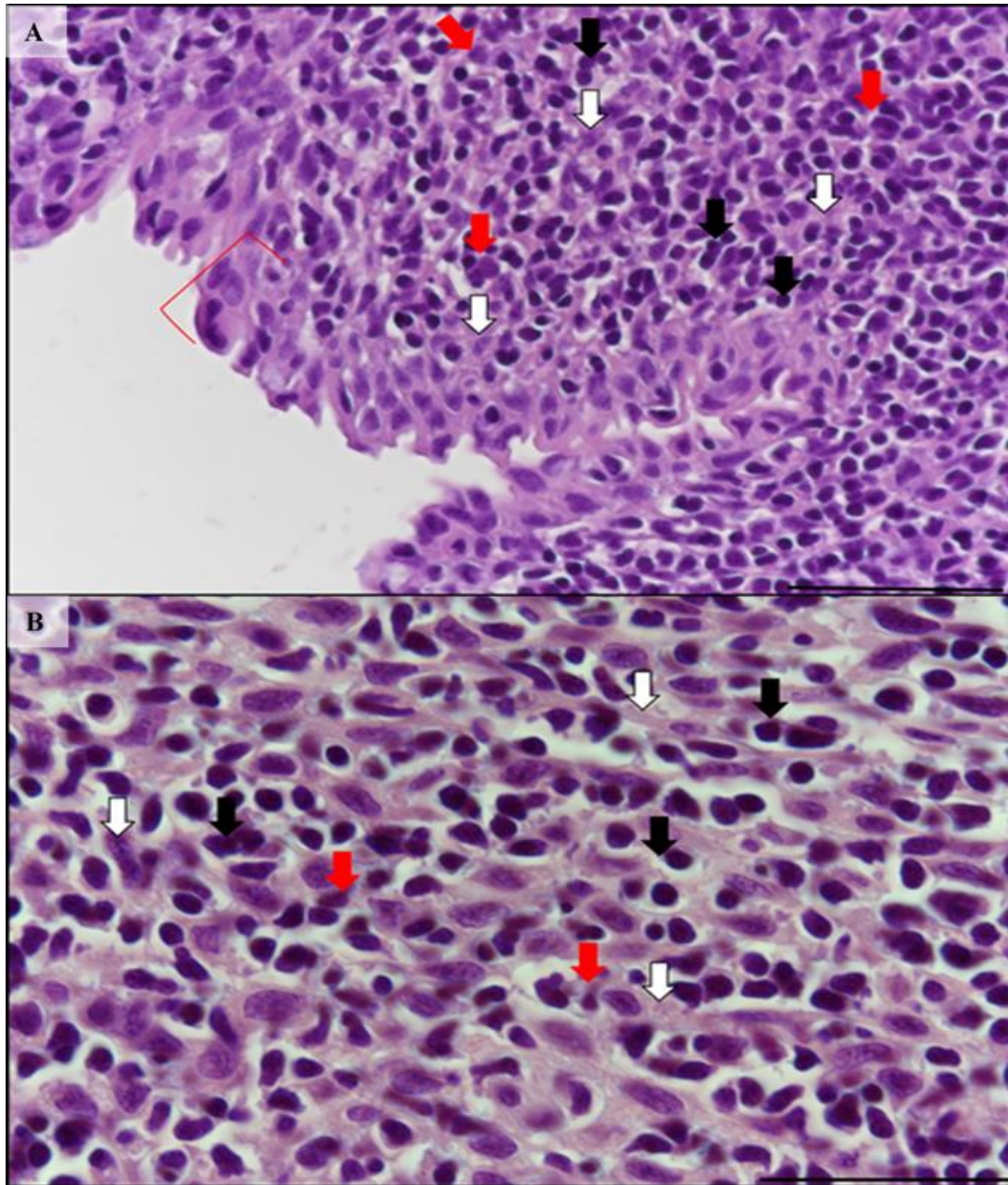


Figure 3.2 Interbranchial lymphoid tissue of Atlantic salmon unexposed to *N. perurans*. (A) pILT with mucous cells apically present (black arrowheads), lymphocytes (white arrowheads) and epithelial cells forming a meshwork (red arrowheads). (B) dILT with mucous cells apically present (black arrowheads), lymphocytes (white arrowheads), epithelial cells forming a meshwork (red arrowheads) and the basement membrane (black arrow).





*Figure 3.3 Distal ILT adjacent to a lesion in a singly exposed (SE) fish, limited by a basement membrane (black arrowheads), epithelial cells presenting stippled chromatin (white arrows), apoptotic cells (white arrowhead) small eosinophilic mononuclear cells (EMNC) (red arrows) and areas presenting oedema (red arrowheads) (H&E, bar=25  $\mu$ m).*



*Figure 3.4 Proximal ILT in a filament that presents a lesion in a repeatedly exposed (RE) fish. (A) The apex of the ILT presented a layer of epithelial cells with a trabecula-like organization creating a capsule-like structure (red line); the epithelial cells had abundant cytoplasm and large nucleus (white arrows). Clusters of lymphocytes were observed (black arrows) and small eosinophilic mononuclear cells (red arrows) (H&E, bar =50  $\mu$ m). (B) Central area of the ILT with abundant lymphocytes (black arrows), epithelial cells (white arrows), small EMNC (red arrows) (H&E, bar=25  $\mu$ m).*

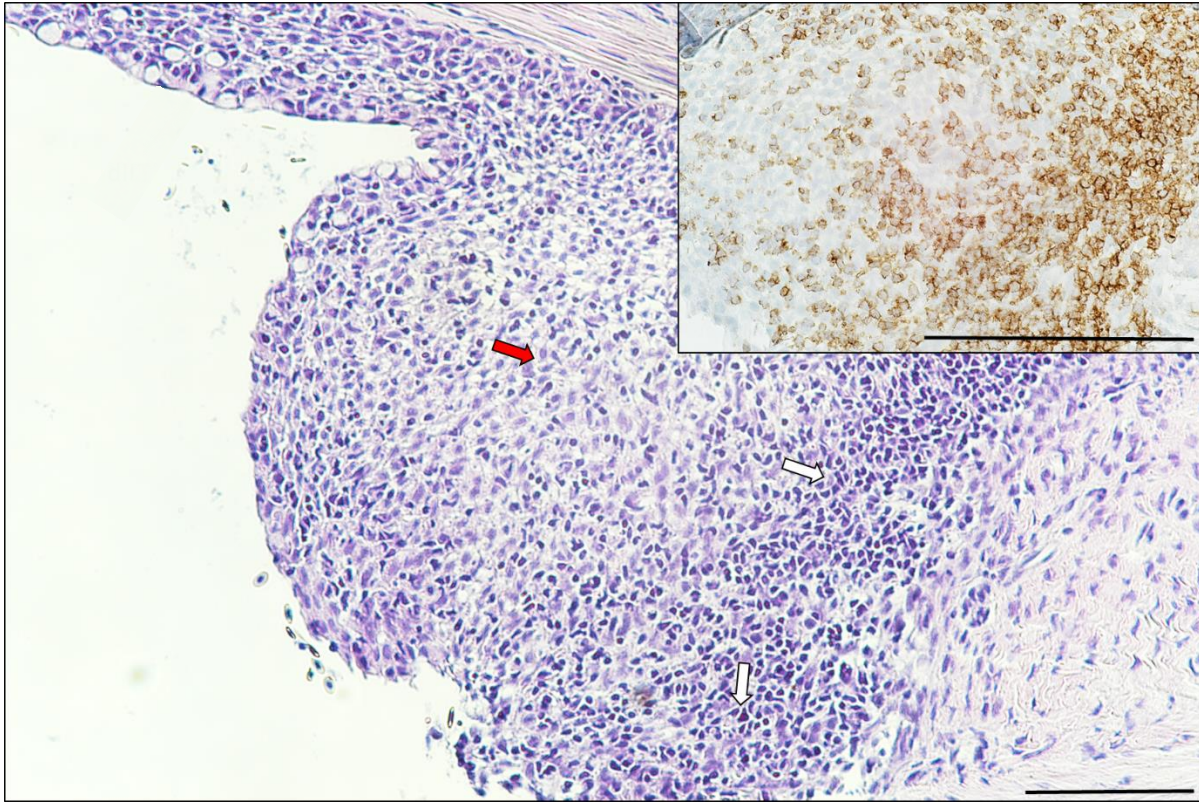
In several sections of AGD affected fish a segregation of lymphocytes was commonly observed in the proximal ILT, with a lower density of lymphocytes in the centre of the ILT, and higher



density in the basal area and the periphery of the structure including the dILT (Figure 3. 5). IHC revealed that the segregated cells were CD3<sup>+</sup> (Figure 3. 5, insert).

Differences in the density of CD3<sup>+</sup> cells were also observed in the dILT close to lesions. In some cases, a high density of CD3<sup>+</sup> cells were observed along the dILT and lesion. In some sections the dILT appeared to be protracting to adjacent lesions with lymphocytes, epithelial cells, scattered small EMNC and mitotic cells present from the trailing edge of the filament to the lesion in the lamellae (Figure 3.6, A, B, C, D). IHC revealed that it was likely that the lymphocytes involved in the possible protracting of the ILT were mostly CD3<sup>+</sup> cells (Figure 3.6, A, insert).

It was observed that in some sections the dILT with an adjacent lesion was enlarged (Figure 3. 7, A) whilst in areas without lesions the dILT had a normal structure and size. IHC for CD3<sup>+</sup> cells revealed that very few T-cells were present in the enlarged the enlarged dILT (Figure 3. 7, A, insert). The majority of the cells in the enlarged dILT were epithelial cells with an enlarged nucleus with stippled chromatin, a large cytoplasm (Figure 3. 7, B, C). Pyknosis was also observed (Figure 3. 7, B, C).



*Figure 3. 5 Proximal ILT in RE fish, with the central area presenting mostly epithelial cells (red arrow) and lymphocytes mostly present in the periphery of the pILT (white arrows). Insert showing IHC stain of CD3<sup>+</sup> cells revealing segregation of these cells in the pILT.*



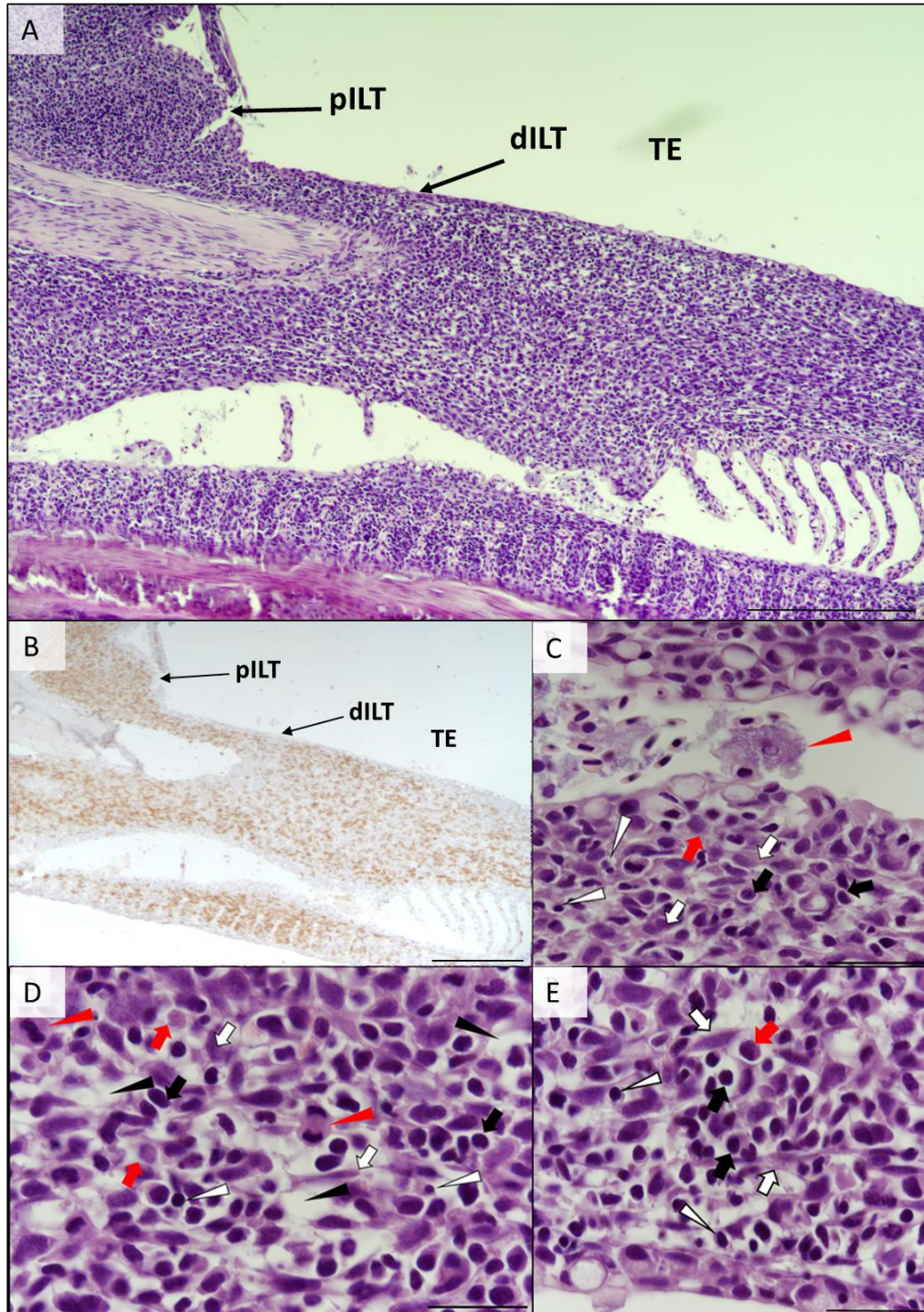


Figure 3.6 Gill from a RE fish showing (A) Transversal section showing the proximal ILT (pILT) and distal ILT (dILT) in the trailing edge of the filament (TE), extending to the AGD lesion (\*) (H&E, Bar = 250  $\mu$ m). (B) IHC stain of CD3<sup>+</sup> cells of the section in (A) showing the proximal ILT (pILT) and dILT (dILT) in the trailing edge of the filament (TE) (IHC, Bar = 250  $\mu$ m). (B) Top of the lesion showing an associated amoeba (red arrowhead). The lesion has scattered lymphocytes (black arrows), small EMNC (red arrows), epithelial cells with abundant cytoplasm (white arrows) and some pyknosis (white arrowhead) (H&E, Bar = 25  $\mu$ m). (C) Cells between the dILT in the trailing edge and the lesion, with

scattered lymphocytes (black arrows), eosinophilic granulocytes (red arrows), epithelial cells with abundant cytoplasm (white arrows) and some pyknosis (white arrowhead). Mitotic cells were observed (red arrowheads) and oedema (black arrowheads) (H&E, Bar = 250  $\mu$ m). (D) dILT in the trailing edge, with lymphocytes (black arrows), small EMNC (red arrows), epithelial cells (white arrows) pyknosis (white arrowhead) (H&E, Bar = 250  $\mu$ m).

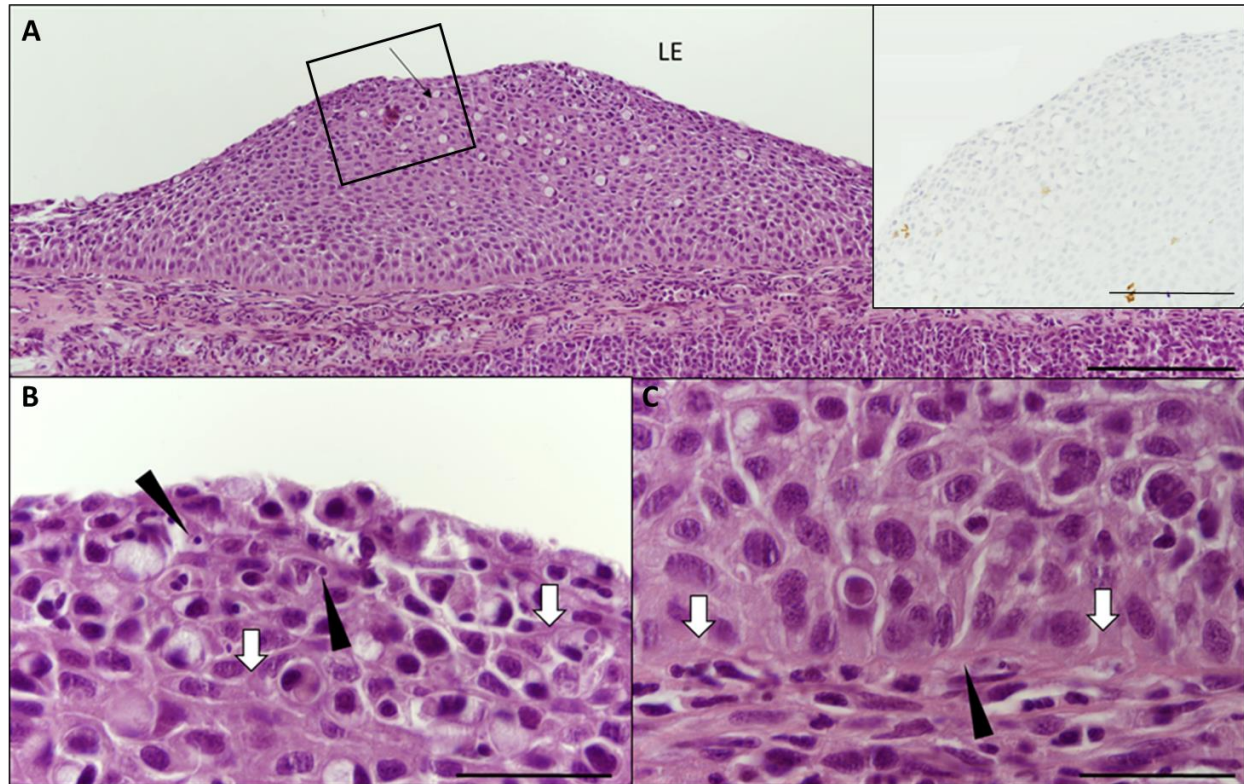


Figure 3. 7 Gill from a SE fish showing (A) Leading edge of the filament (LE) with an enlarged dILT (arrow) (H&E, Bar=250). Insert showing immunohistochemical detection of CD3 revealing very few positive cells in the enlarged ILT (B) Apex of the enlarged dILT revealing stromal cells with an abundant cytoplasm (white arrows) and pyknotic cells (arrowheads) (H&E, Bar=25). (C) Basal area of the enlarged dILT revealing a prominent basal membrane (arrowhead), and epithelial cells with abundant cytoplasm forming a palisade-like structure (white arrows).

### 3.4.2 Cell Quantification and Area Measurement

The presence of adjacent lesions, but not exposure, was found to have a significant effect on the number of CD3<sup>+</sup> cells in the pILT ( $F= 13.22$ ,  $df= 1$ ,  $p= 0.001$ ). The number of CD3<sup>+</sup> cells in the pILT with adjacent lesions was significantly higher (Mean= 83.37, SEM= 4.07) compared to pILT sections with no adjacent lesions (Mean= 63.08, SEM= 3.81) (Figure 3.8, A). No significant effects of lesion presence ( $F= 0.930$ ,  $df= 1$ ,  $p= 0.339$ ) and exposure ( $F= 0.005$ ,  $df= 1$ ,



$p = 0.947$ ) were observed for the number of CD3<sup>-</sup> cells in the pILT (Figure 3.8, B). A significant effect of both lesion presence ( $F = 12.22$ ,  $df = 1$ ,  $p = 0.001$ ) and exposure ( $F = 4.49$ ,  $df = 1$ ,  $p = 0.048$ ) was found for total number of cells in the pILT (Figure 3.8, Ci and Cii). The total number of cells in the pILT with adjacent lesions was significantly higher (Mean = 118.20, SEM = 3.57) compared to the pILT without adjacent lesions (Mean = 101.08, SEM = 3.49). Similarly, the total number of cells in the pILT of repeatedly exposed fish was significantly higher (Mean = 114.60, SEM = 4.21) compared to naive fish following a single *N. perurans* exposure (Mean = 104.68, SEM = 4.86). No significant effects of lesion presence or exposure were observed for area ( $F = 0.214$ ,  $df = 1$ ,  $p = 0.646$ ) (Figure 3.8, D). Evaluation of number of lesions in sagittal sections revealed that a higher variability was observed in repeatedly exposed fish when compared with singly affected fish (**Error! Reference source not found.**). Correlation analyses for % AGD severity, pILT area, number of CD3<sup>+</sup> cells, number of CD3<sup>-</sup> cells and number of total cells found a significant moderate negative association between the number of CD3<sup>+</sup> and CD3<sup>-</sup> cell numbers ( $r = -0.979$ ,  $n = 31$ ,  $p < 0.001$ ) (Figure 3. 9). No other correlations were found to be significant (data not shown).

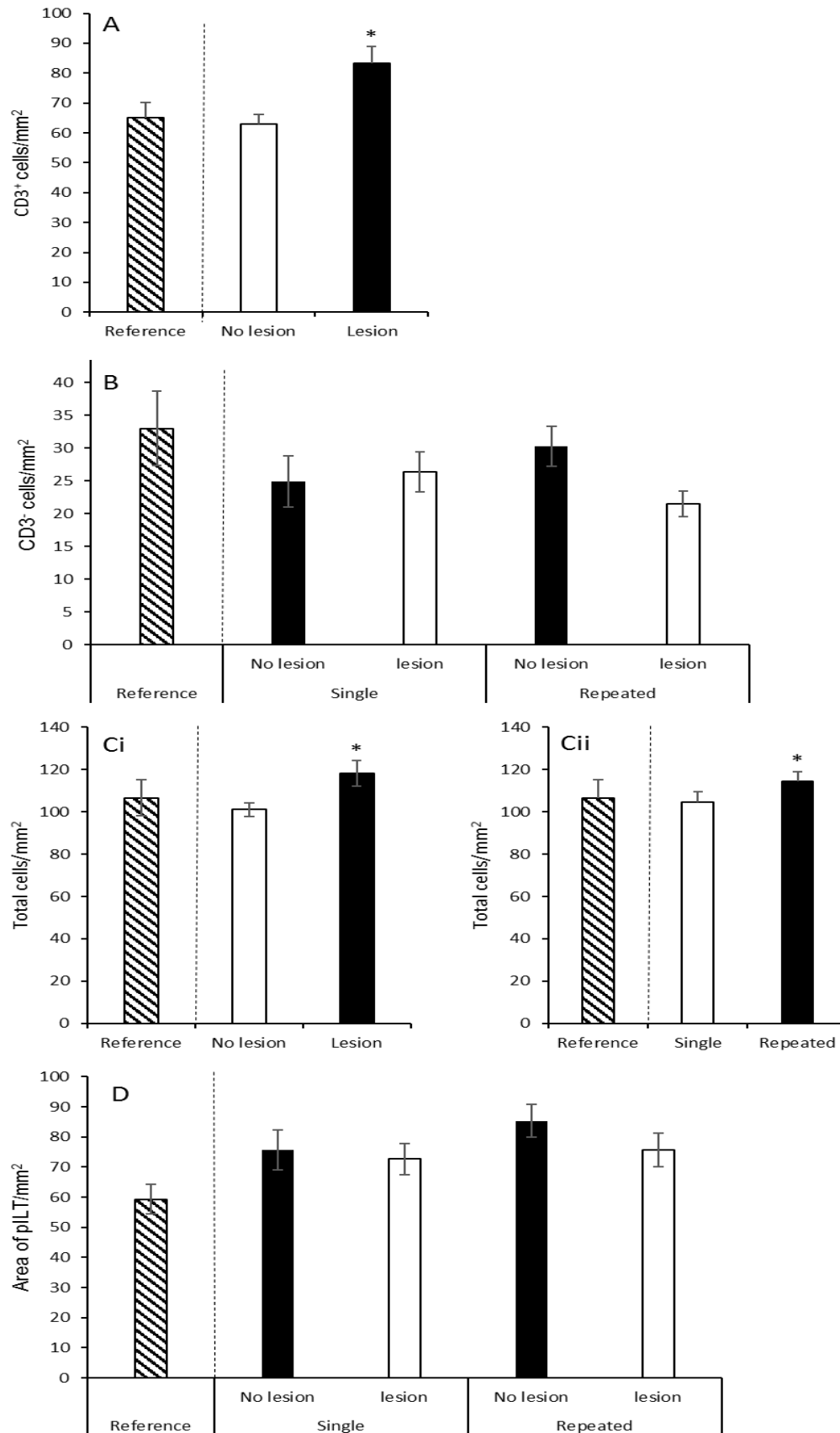


Figure 3.8. Effects of *N. perurans* exposure (single ( $n=18$ ) or repeated ( $n=40$ )) and AGD lesion presence (lesion ( $n=22$ ) or no lesion ( $n=36$ )) on the number of (A)  $CD3^+$  cells in the pILT, data grouped according to lesion presence, (B)  $CD3^-$  cells (C) total cell numbers in the pILT (positive, negative and ambiguous), data grouped according to lesion presence (Ci) and exposure (Cii) and (D) total area of the pILT in AGD affected Atlantic salmon. Unexposed fish data ( $n=13$ ) was used as a reference but was not included in the ANOVA analyses. Values are mean  $\pm$  SEM. (\*) indicates values that are significantly different between groups  $p < 0.05$ .

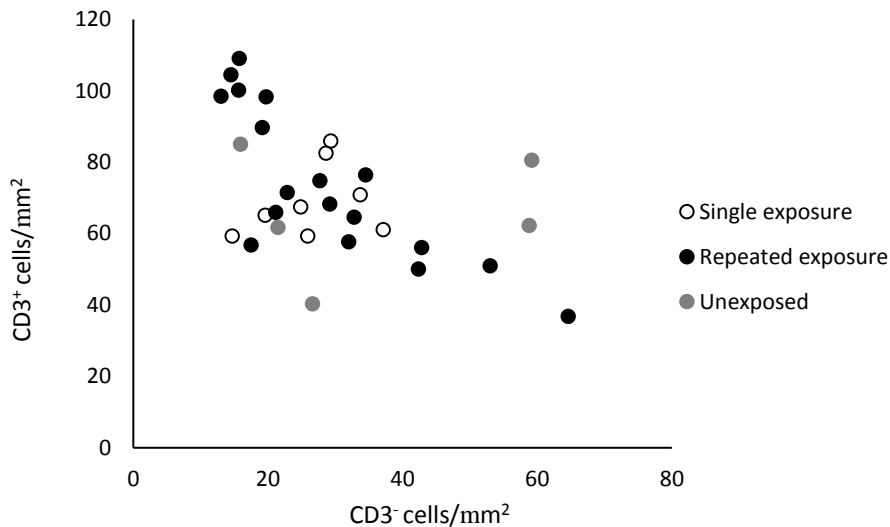


Figure 3.9 Relationship between  $CD3^-$  and  $CD3^+$  cell numbers/mm<sup>2</sup> of pILT. The correlation using all data was significant ( $r = -0.979$ ,  $n = 31$ ,  $p = 0.00$ ).

### 3.5 Discussion

The results from this study demonstrated that AGD induces changes in the T-cell population of the ILT close to lesions. The higher number of  $CD3^+$  in the pILT adjacent to lesions may indicate that inflammation is occurring, and it is possible that the T-cells present in the pILT are recruited to lesion sites. The histopathological assessment in this study corroborates this observation.

Previous research using histology has described three stages of AGD pathology starting with a noticeable innate cellular immune response (Adams and Nowak, 2003). This response was characterized by the presence of a large number of macrophages and neutrophils throughout the hyperplastic lesions (Adams and Nowak, 2003). In the present study, the presence of high numbers of  $CD3^-$  cells may indicate the presence of B-cells ( $CD8^+$  cells). However, Dalum et al., 2015 revealed that few  $CD8^+$  cells were present in the ILT of healthy Atlantic salmon. It is

likely that the CD3<sup>-</sup> cells may be cells belonging to the first phase of the cellular immune response, similarly to the ones observed in Adams and Nowak (2003). Further studies analysing the presence of CD8<sup>+</sup> cells in the ILT of AGD affected Atlantic salmon will be of high importance to understand the cellular immunity in this tissue. The second stage of AGD pathology is characterized by leucocyte infiltration (Adams and Nowak, 2003). Adams and Nowak, 2003 did not identify the leucocytes observed during the second stage however results from the present study suggest a high proportion of the leucocytes were T-cells (CD3<sup>+</sup> cells).

During the final stage of AGD it was observed that larger mature lesions presented a stratified squamous arrangement of epithelial cells with a low number of leucocytes (Adams and Nowak, 2003). Furthermore, analysis of gene expression revealed that there was a down-regulation of genes involved in antigen processing and presentation during AGD (Young, et al. 2008b). This down regulation was restricted to sites of AGD lesions (Young, et al. 2008b). A previous study also found a reduction of lymphocytes in the pILT of AGD affected fish at day 28 post infection (Norte dos Santos et al., 2014, Chapter 2). Previous research revealed that in murine amoebic colitis, caused by *E. histolytica*, the acquired T-helper cell (CD4) response aggravates disease (Haupt et al., 2002). The same study revealed that depletion of T-helper cells further diminished the burden of *E. histolytica* and the inflammation. This may also be occurring in the gills of AGD affected fish. It has been established that the majority of the immune cell population in the ILT are CD3<sup>+</sup>, in which a small subset are cytotoxic T-cells (CD8<sup>+</sup>), and T-helper cells (CD4<sup>+</sup> cells) (Dalum et al., 2015). In the present study, it is possible that an infiltration of CD3<sup>+</sup> cells occurred next to lesions. Further study on the identification of cells in both the ILT (proximal and distal) and AGD lesions will help understanding the role of the ILT in the AGD response in terms of susceptibility and resistance. This knowledge would provide much greater insight into AGD related mucosal immunity.

*N. perurans* Histopathological observations revealed that the dILT presented a higher density of CD3<sup>+</sup> cells when close to AGD lesions containing a high number of CD3<sup>+</sup> cells. The histopathological changes appeared to be more extreme in the singly affected fish than in the repeatedly exposed fish. The major difference between repeatedly and singly exposed fish was the restructuring of the tissue, with a lower number of CD3<sup>+</sup> cells (also observed in the quantitative assessment) in the singly exposed fish. In the most AGD affected areas the cellular



structure of both proximal and distal ILT presented enlarged epithelial cells with an enlarged nucleus and abundant cytoplasm. An apparent diffuse infiltration of CD3<sup>+</sup> cells from the dILT to the lesions was observed in some lesions. It has been suggested that the ILT works as an immunosurveillance tissue, with the pILT acting as a reservoir of T cells which can migrate through the dILT to the location of antigen exposure (Koppang et al., 2010). Furthermore, CD3<sup>+</sup> cells were observed close to the filament below the pILT. It was also observed that the IHC stain was more intense in this area when a lesion was present. This may indicate that lymphocytes migrate from the pILT down the filament to access lesions or it is possible that this is an extension of the ILT, similar to the dILT. However, it is also possible that cellular proliferation characteristic of AGD lesions is extending towards the ILT. More studies focusing on the cells present below the pILT will be important to understand the distribution of the ILT in other gill regions. An extension of the ILT towards the basal area of the filament would facilitate the surveillance and T-cell migration within not only the distal area, but also in the basal area of the gill arch. Furthermore, it was suggested that the close association between the dILT and the afferent arterioles may be the route that the T-cells migrate between the ILT and other tissues. This may represent the connection between mucosal and systemic immunity (Dalum et al., 2015; Rasmussen et al., 2013).

In this study, it was found that a higher variability in the number of lesions within groups were in the repeatedly exposed fish, possibly due to the differences in susceptibility to AGD after previous exposures. Adams et al., 2016 suggested that under certain conditions fortification of lesions during later stages of disease as described in Adams and Nowak (2003) could be a refractory host measure. However, further investigation is needed to further understand the individual differences observed within groups. The only significant effect of exposure, either single or repeated, was found for total number of cells in the pILT although there was no significant difference in the surface area of the pILT suggesting an increase in cellular density occurred in fish repeatedly exposed to *N. perurans*. How this relates to T cells is unclear as a similar significant effect was not observed for the CD3<sup>+</sup> or CD3<sup>-</sup> cell numbers despite the negative correlation found between these cell populations. Previous research found that the pILT of fish affected by ISA-virus decreased in size in the latter stages of the infection (Austbo et al., 2014). In the same study, it was hypothesized that T-cells could be migrating from the ILT to locations exposed to antigens. This migration would lead to the decrease in the size of the pILT.

In the ILT of fish affected by AGD an increase in the size of the pILT of affected fish was observed as well as a decrease in lymphocyte density in fish presenting heavy severity (Norte dos Santos et al., 2014, Chapter 2). However, caution is advised when comparing a viral disease with a parasitic disease, since in AGD proliferation is a characteristic of the lesions. It was suggested that proliferation of epithelial cells could be causing the increase in the size of the ILT in naïve fish affected by AGD at 28 days post infection (Norte dos Santos, 2014, chapter 2). Another possibility is that the ILT had reached morphostasis (tissue homeostasis). It is known that the immune system has an important role in tissue morphostasis inducing tolerance to self-antigens within the adaptive immune system by deleting autoreactive T and B lymphocytes (Conrad et al., 2007; Mueller, 2010). However, tissue morphostasis is a very complex process (Bukovsky 2011), and further work is required to know if this process occurs in the ILT.

### 3.6 Conclusion

The proximity of lesions is the major factor affecting the cellularity of the pILT in fish affected by AGD. An increase in cellularity may indicate recruitment of immune cells to lesion sites. Furthermore, a higher number of cells was observed in the pILT of repeatedly exposed fish, suggesting a stronger immune response to AGD. Furthermore, histopathological assessment revealed that a more extreme restructuring was observed in both distal and proximal ILT of singly affected fish, compared with the repeatedly exposed. Further studies focusing on additional cell types will be of high importance to understand which cells are present in the different stages of this disease, and their possible functions. This study has contributed to the understanding of the cellular response during AGD. It may shed some light in regards to ILT function. In the future, this organized MALT may be used as a target for vaccination.

# Chapter Four

## Southern Bluefin Tuna (*Thunnus Maccoyii*)

### Gill-Associated Mucosal Lymphoid-Like Tissue

#### 4.1 Introduction

The gills in tunas are modified to maximize the intake of oxygen, which is critical to support their active lifestyle (Olson et al., 2003). Tunas are ram ventilators, and the morphometrics of the gills are adapted to combine elevated energetic demands (Wegner et al., 2010). Compared to those in other teleosts, the lamellae in tunas are longer with short widths. Thus the filaments bear a high lamellar frequency (Wegner et al., 2010). Furthermore, to meet the requirements of ram ventilation, the tips of the lamellae are fused, providing increased flow resistance of the gills (Wegner et al., 2013).

It has been shown that tuna gills that are affected by parasites mount an immune response (Mladineo and Block 2010, Polinski et al., 2014). Infection with *Didymosulcus katsuwonicola* (Digenea) induced an immune response in the gills of Atlantic bluefin tuna (*Thunnus thynnus*) with up-regulation of pro-inflammatory cytokines IL-1 $\beta$  and TNF $\alpha$ 2 by pathogen-associated molecular patterns (PAMPs). Furthermore, a tissue response with a strong presence of mast cells, lymphocyte-like cells, eosinophils and occasional rodlet cells was observed during the infection (Pleic et al., 2015). It has been suggested that tuna have a well-adapted innate immunity in the gills (Mladineo and Block, 2010). The up-regulation of pro-inflammatory cytokines occurred in the *Didymocystis wedli* location, rather than a systemic response (Mladineo and Block 2010). The cytokine expression in Pacific bluefin tuna (*Thunnus orientalis*) affected by *D. wedli* suggested that fibroblasts and lymphocytes may be the cells responsible for cytokine secretion in the vicinity of cysts in the gills (Mladineo and Block 2010). The presence of a stratified squamous epithelium with mast cells and eosinophils, supported by a basement

membrane, was observed close to the didymozoid hind body in the gills of Atlantic bluefin tuna (Pleic et al., 2015). This may indicate that some resident immune cells may be present in the tuna gills, and migrate to the sites of parasite attachment.

The evidence of immune reactivity of the tuna gills suggests the possibility that tuna gills possess a resident population of immune cells. A lymphoid tissue was recently described in the gills of salmonids, and named interbranchial lymphoid tissue (ILT). The ILT is located on the caudal edge of interbranchial septum at the base of the gill filaments and extends along each side of the gill filament epithelium in the Atlantic salmon (Haugarvoll et al., 2008). The ILT is divided into the proximal ILT, located in the very terminal end of the interbranchial septum, and the dILT, located along the trailing edge of the lymphoid tissue (Dalum et al., 2015). Both proximal and dILT present the same morphological and immunohistochemical characteristics and consist of a continuous network of epithelial cells that incorporate a large population of T cells limited basally by a basement membrane. The cells in the ILT are mostly CD3<sup>+</sup> cells, being the majority T-helper cells (CD4<sup>+</sup> cells) and in lower numbers cytotoxic T cells (CD8<sup>+</sup>). MHC class II and TCR complex are also present in the ILT (Aas et al., 2014; Dalum et al., 2015; Koppang et al., 2010). The presence of these molecules is the most important for the T cell immune response, which allows the recognition of infected cells, and MHC class I and II for recognition of antigen-presenting cells (Fischer et al., 2013). Thus, the ILT is considered an important structure for the induction of T cell-mediated immunity and has a strategic position regarding antigen encounter (Aas et al., 2014). It is likely that the resident population of immune cells are a similar structure of the ILT observed in salmonids. This study aimed to determine if a cellular structure resembling GIALT is present in apparently healthy southern bluefin tuna (SBT) (*Thunnus maccoyii*).

## 4.2 Materials and Methods

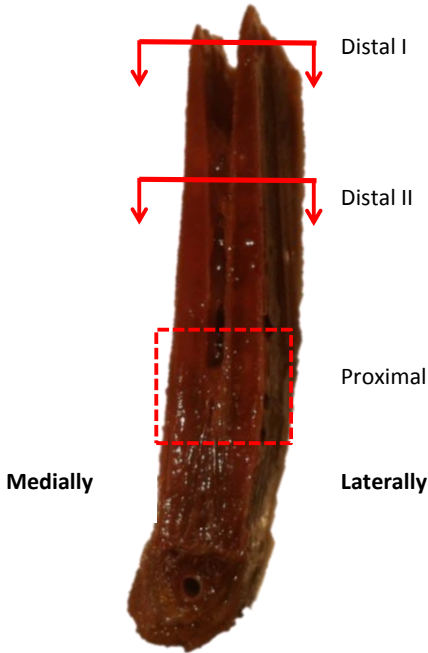
### 4.2.1 Sample Collection

Wild SBT(n=20, mean weight: 22.1kg) were caught by rod and reel at Pedra Branca, Dover, Tasmania (43°51'36"S 146°58'28"E). A section (approximated 5mm<sup>2</sup>) of the first gill arch was cut on the boat and placed on ice in 3% glutaraldehyde (CH<sub>2</sub>(CH<sub>2</sub>CHO)<sub>2</sub>) in a 0.1M cacodylate buffer (pH 7.4) for 5-8 hours for transmission electron microscopy. The rest of the gill basket

was collected and kept on ice until processed inshore where sections of the gills were cut and immersed in 10% neutral-buffered formalin for 24 hours prior to further histological processing.

### *4.2.2 Light Microscopy*

A section of the gill comprising 4–6 filaments was cut and transversely oriented in a histological cassette according to (Haugarvoll et al., 2008). Gills were decalcified for one hour using rapid decalcifying fluid (Australian Biostain Pty. Ltd.) and then processed routinely for histology. For observation of the dorsal sections, a gill section consisting of 4–7 filaments was embedded in paraffin and the regions of interest were excised using a blade (*Figure 4.1*). The sections were then oriented in the dorsal plane and re-embedded. Sections 5- $\mu$ m-thick were cut and allowed to dry overnight at 37°C before being dewaxed and dehydrated. To assess the cells in the transversal and dorsal sections by light microscopy, sections were stained with hematoxylin and eosin (H&E) and Periodic acid Schiff with hematoxylin counterstain. To reveal the basement membrane, sections were stained with periodic acid-Schiff stain and then dehydrated in a graded bath of ethanol and cleared in xylene. All sections were analyzed using an Olympus BH-2-RFCA (Japan) compound light microscope.



*Figure 4.1.* Transverse orientation of the gill arch of southern bluefin tuna (SBT) (macroscopic view). Red lines indicate the area where dorsal sections were cut to observe GIALT in the dorsal plane (arrows represent the orientation of sectioning). The dashed box represents the section oriented transversally for observation of the interbranchial septum.

### *4.2.3 Immunohistochemistry*

Anti-piscidin 3 antibody (anti-HAGR) (Bethyl Laboratories) was used (pre-diluted in PBS), following the method of Andrews et al. (2010). Five- $\mu$ m sections from the above-mentioned samples were cut and mounted on poly-L-lysine-coated slides (Superfrost® Plus, Thermo Scientific, D-38116 Braunschweig, Germany). Sections were dewaxed in xylene, hydrated in graded alcohol to distilled water. Samples were blocked for endogenous peroxidase activity in 3%  $\text{H}_2\text{O}_2$  for 10 minutes (Li et al., 1987), rinsed in water, and washed with phosphate-buffered saline (PBS, pH 7.2). Samples were incubated with a 1:400 dilution of the primary antibody (anti-HAGR) for 30 minutes at room temperature, washed with PBS and incubated with a secondary antibody anti-rabbit IgG conjugated with horse radish peroxidase (Sigma-Aldrich, Australia) for 30 minutes, then rinsed in PBS and incubated with DAB substrate chromogen for 5 minutes. Following counterstaining with Mayer's hematoxylin for 30 seconds, dehydrated in a series of ethanol rinses and mounted in DPX. Negative control reagent (Dako, Australia) was

used as a negative control, and the intestines of striped bass and Atlantic salmon were used as positive controls.

For cytokeratin immunohistochemistry heat induced epitope retrieval was done by autoclaving the slides in citrate buffer (pH 6.0). Immunohistochemical stain was done as per procedures of a commercial kit (Dako, Australia). Sections were incubated for 60 minutes with a commercial pre-diluted primary antibody Anti-Cytokeratin AE1/AE3 (Pan cytokeratins) clone AE1/AE3 (IHC Select® No. IHC2025-6, © Merck KGaA, Darmstadt, Germany). Following incubation, the sections were washed in PBS followed by incubation with peroxidase labelled polymer for 5 minutes then washed again in PBS. Substrate chromogen (DAB) was added to each section and after 5 minutes followed by distilled water immersion to stop the reaction. Slides were then immersed in hematoxylin (10 dips), washed in water and bluing was done in Scott's tap water. Finally, the sections were dehydrated and mounted in DPX.

#### *4.2.4 Transmission electron microscopy (TEM)*

Samples for TEM (n=5, approximately 5 mm by 3 mm) were excised from the interbranchial septum and fixed according to the two-stage fixation technique. After the primary fixation in 3% glutaraldehyde, samples were washed thoroughly with the buffer solution 0.1M cacodylate buffer (pH 7.4). Samples were post-fixed in 2% osmium tetroxide OsO<sub>4</sub> and 1.5% potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] in a 0.1M cacodylate buffer (pH 7.2) for 1 hour. To enhance image quality, *en bloc* staining was achieved by immersing samples in 1.5% uranyl acetate (UO<sub>2</sub>(CH<sub>3</sub>COO)<sub>2</sub>), and then samples were dehydrated through a series of alcohols. Semi-thin (1 µm sections) were cut, mounted on glass slides and stained with toluidine blue. Ultrathin sections of LX 112 resin-embedded (Brandtzaeg) gill samples were placed on grids and post-stained using 1.5% uranyl acetate in a petri dish followed by 3% lead citrate solution by adding a drop in the petri dish and placing the grid (section place down) to float in the stain for 2-3 min (Ellis, 2007). Beakers were filled with freshly boiled deionized water (warm), and the grids were swirled in the water, followed by drying with filter paper. After processing, a limited number of sections were obtained, thus it was not possible to visualise all the cells present in the tissue.

## 4.5 Results

The dorsal section of the gill arch revealed a continuous structure composed of cells in the interbranchial septum below the gill epithelium extending in the trailing edge of the filament (Figure 4.2, A). This cellular structure was present in the interbranchial septum (Figure 4.2, B) and along the trailing edge of the gill filament (Figure 4.2, C). In some sections the lymphoid cell structure of the trailing edge presented a sinuous structure with bulbous protuberances (not shown). A basement membrane was present, separating the cell accumulation from the gill remaining. The cellular accumulation was mostly composed of lymphocytes and eosinophilic granulocytes and mucous cells were observed apically (Figure 4.2, B, C).

A high magnification of the lymphoid structure revealed that it consisted of intra-epithelial lymphocytes and eosinophilic granulocytes (EGs) (Figure 4.3, A). Two different types of EGs were observed, one with markedly eosinophilic cytoplasm, with distinguishable granules in the cytoplasm and a peripherally-located nucleus, while the other presented a lighter cytoplasmic coloration and the nucleus was elongated (Figure 4.3, A). Epithelial-like cells were present in the cellular accumulation, and were more obvious close to the basement membrane, extending towards the apex of the cell accumulation (Figure 4.3, A). Transmission electron microscopy revealed the presence of lymphocytes in the cell accumulation, presenting pseudopodia (Figure 4.3, B). Epithelial cells were present closely associated with the lymphocytes (Figure 4.3, B). Piscidin 3 antibody revealed that the eosinophilic granulocytes expressed this antimicrobial peptide, with two seemingly different EG types presenting different intensity of IHC reaction (Figure 4.3, C). IHC with cytokeratin antibody revealed a meshwork of epithelial cells, with a stronger staining in the apical area of the cell accumulation (Figure 4.3, D).

A low magnification view of a dorsal section of the gill arch revealed the structures of the leading and trailing edges of the filament (Figure 4.4, A). The trailing edge of the filament presented a cellular structure located mainly in the lateral areas of the afferent filamental artery and appeared to connect the edges of the adjacent filaments. This was not observed in the leading edge of the filament, where the structure was more compacted. The lamellar fusion was also observed in the dorsal sectioning, and it was observed that this structure presented a cell accumulation similar to the one observed in the filaments (Figure 4.4, A, insert). A more detailed observation of the leading edge revealed a dense stroma in the most apical layer, with abundant



mucous cells closer to the surface (Figure 4.4, B). Below the stroma scant lymphocytes and EGs, which the majority were PAS-negative, were observed (Figure 4.4, B). A thick basement membrane was observed bordering the cell accumulation (Figure 4.4, B). In the trailing edge, epithelial cells formed a less dense structure when compared with the leading edge (Figure 4.4, C). A basement membrane separated the cell accumulation from the rest of the gill, and scarce mucous cells were observed in the apex of the cell accumulation (Figure 4.4, C). The majority of the EGs were negative for PAS-staining.

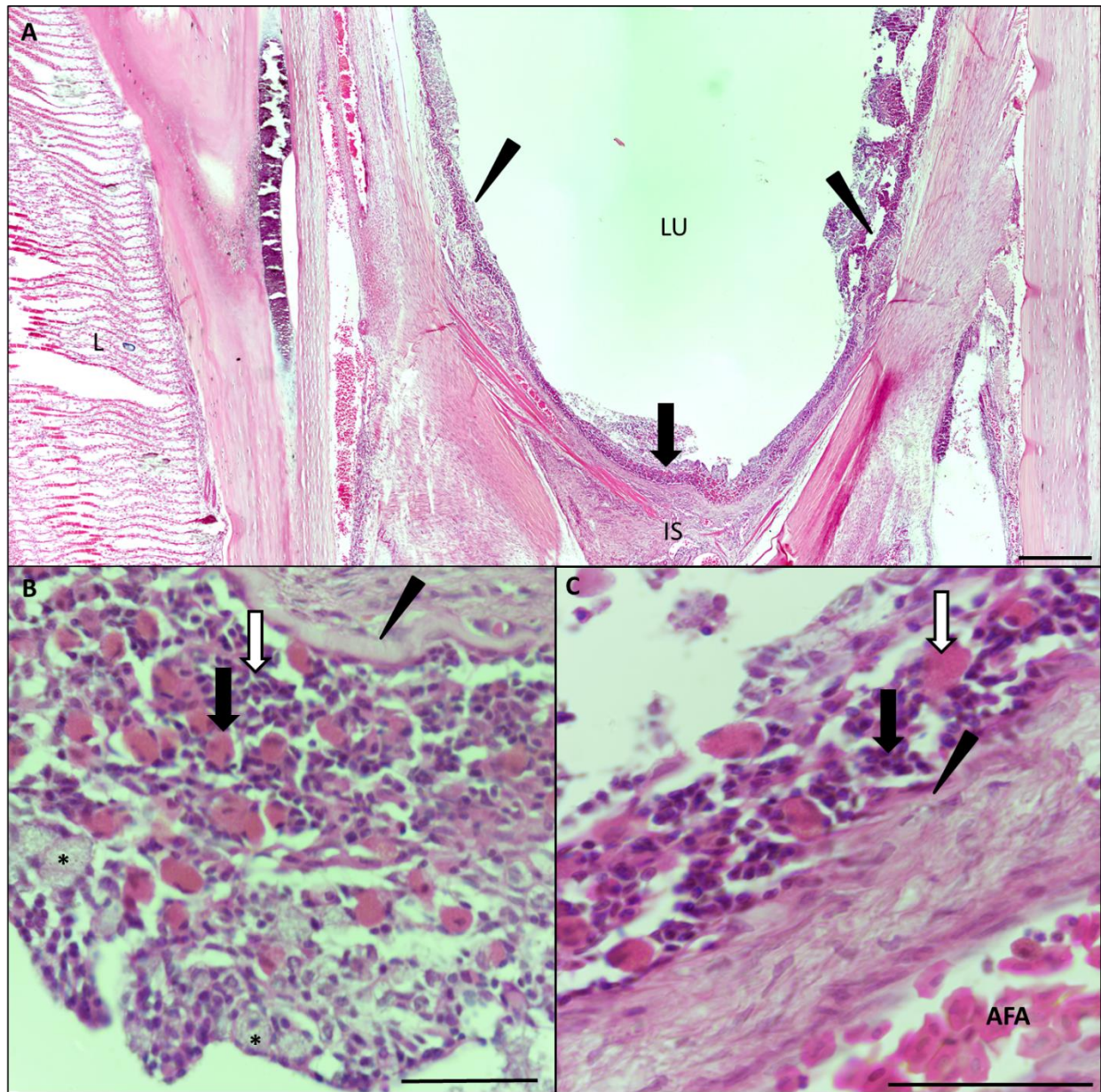


Figure 4.2. (A) Transverse section of the gill showing the lumen of the branchial chamber (Lu), the interbranchial septum (IS) and an area where the accumulation of cells was observed (black arrow), which extends in the trailing edge of the filament (arrowheads) (H&E, bar = 500  $\mu$ m); (B) Detail of the cell accumulation in the interbranchial septum, which comprises a basement membrane (black arrowhead), agglomeration of lymphocytes (white arrow) and eosinophilic granulocytes (black arrow) (H&E, bar = 25  $\mu$ m) (C) Detail of the tissue in the trailing edge of the filament with agglomeration of lymphocytes (black arrow) and eosinophilic granulocytes (white arrow) and connective tissue (black arrowhead) separating the lymphoid cell accumulation and the afferent filamentary artery (AFA) (H&E, bar = 25  $\mu$ m).



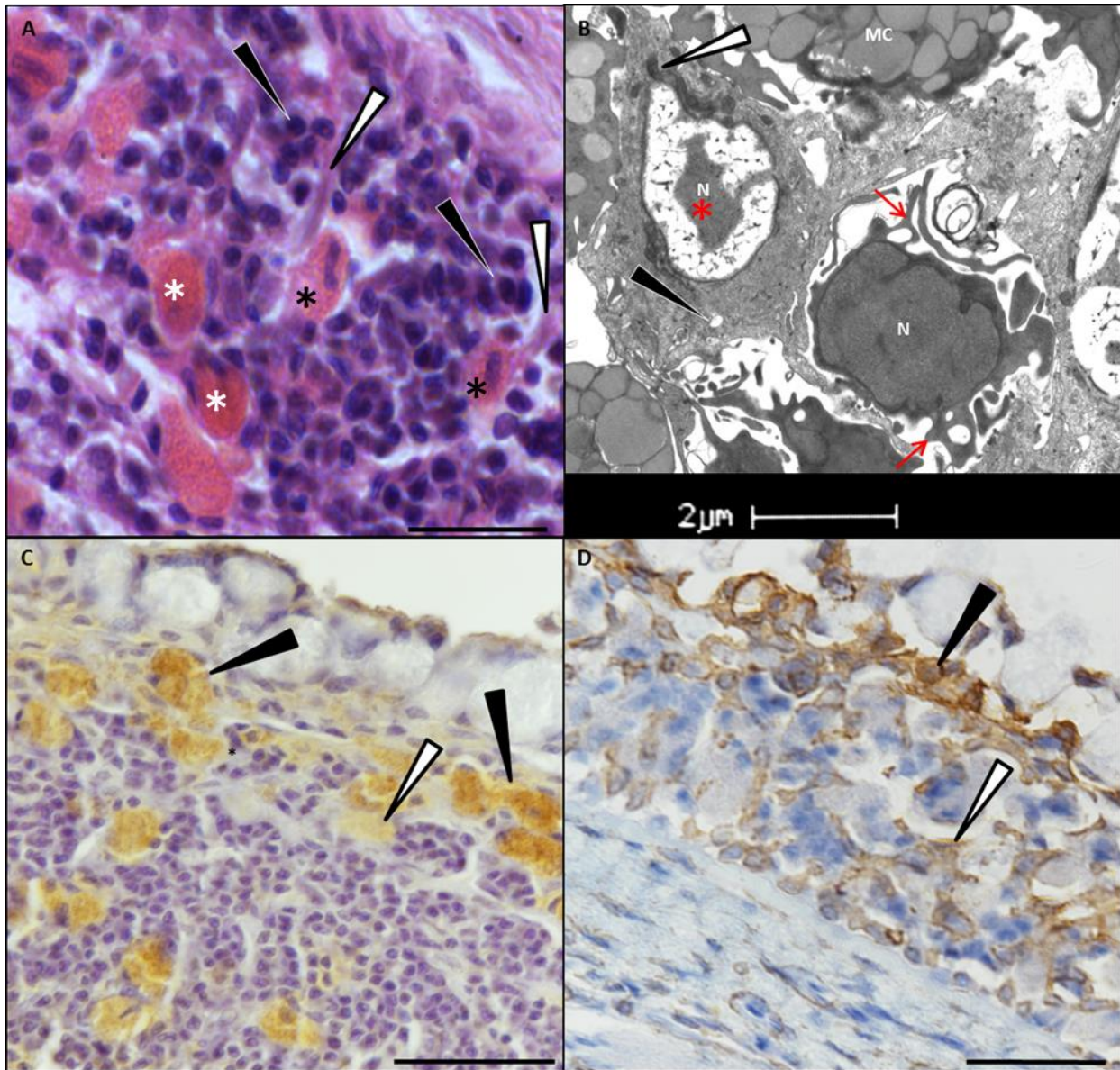


Figure 4.3. Detail of the lymphoid cell agglomeration observed in the interbranchial septum of the gills of SBT. (A) The composition of the cell agglomeration in the interbranchial septum showing lymphocytes (black arrowheads), eosinophilic granulocytes (\*): some presenting elongated nucleus (black \*), and other presenting a marginalized nucleus (white \*) and epithelial cells (white arrowhead) (H&E, bar = 25  $\mu$ m). (B) Transmission electron micrograph of the area showing a reticular epithelial cell (\*) with a large nucleus (N), vacuoles (black arrowhead), and mitochondria (white arrowhead), closely associated with a lymphocyte-like cell presenting large nucleus (N) and pseudopodia (red arrows) (C) Multi-granular cells immunopositive for piscidin 3 with some cells showing a stronger intensity of IHC reaction (black arrowheads) and some with mild intensity of IHC reaction (white arrowhead) (DAB substrate, bar = 25  $\mu$ m) (D) Cytokeratin positive cells (white arrowhead) revealing the meshwork of epithelial cells with more intense staining observed in the apical area of the cell agglomeration (black arrowhead) (bar = 25  $\mu$ m).



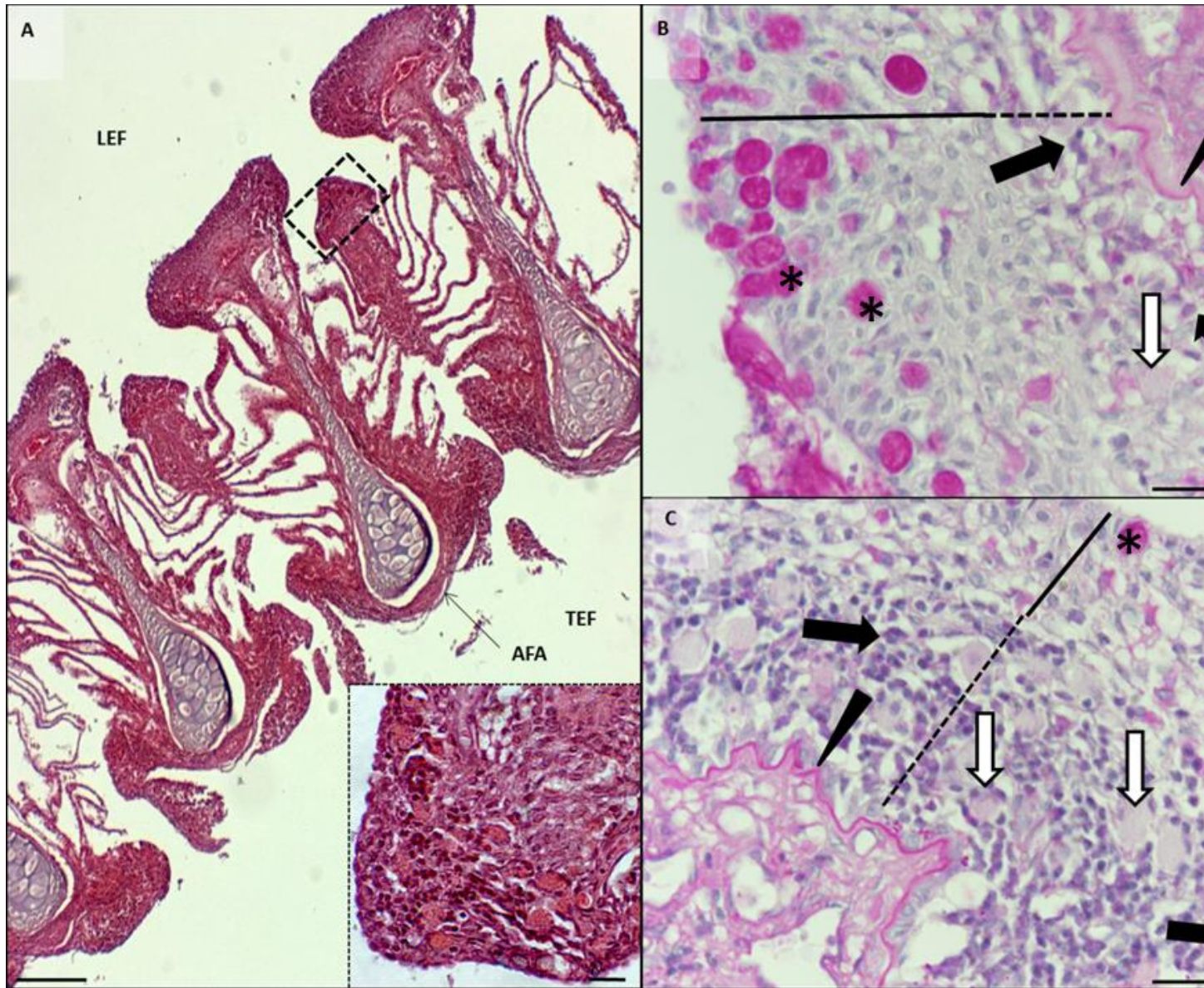


Figure 4.4. Dorsal view of the distal portion of the filament. (A) Dorsal section of the gill showing the leading edge of the filament (LEF), the trailing edge of the filament (TEF) and the afferent filamental artery (AFA). The lamellae fusion is present between filaments (box) and presents a cellular accumulation in the most apical area (insert) (H&E bar = 125  $\mu$ m, bar in insert = 25  $\mu$ m). (B) Leading edge of the filament, with a dense layer of epithelial cells forming a trabecula-like structure (black line) with mucous cells at the surface of the structure (\*). Dashed black line reveals the area with the lymphoid cell accumulation composed of scattered lymphocytes (black arrows) and eosinophilic granulocytes (white arrows). The structure was bordered by a prominent basement membrane (black arrowhead) (PAS-H bar = 25  $\mu$ m). (C) Trailing edge of the filament, revealing a layer of epithelial cells (black line) with scattered mucous cells at the surface of the structure (\*). Dashed black line reveals the area with the lymphoid cell accumulation composed of agglomerations of lymphocytes (black arrows) and eosinophilic granulocytes (white arrows). The structure was bordered by a basement membrane (black arrowhead) (PAS-H bar = 25  $\mu$ m).

## 4.6 Discussion

The present study described a lymphoid accumulation in the gills of SBT, which to the best of my knowledge, has not been described before and likely represents a gill-associated lymphoid tissue resembling the one reported in salmonids (Haugarvoll et al., 2008).

The lymphoid accumulation in SBT was located in the interbranchial septum and extended to the filament epithelium, in a similar way to the ILT in salmonids (Haugarvoll et al., 2008, Dalum et al., 2015). It was composed mostly of eosinophilic granular cells and lymphocytes embedded in a meshwork of epithelial cells with mucous cells present in the external layer. The structure of the lymphoid accumulation was like the one observed in the gills of Atlantic salmon, apart from the high number of EGCs (Haugarvoll et al., 2008, Dalum et al., 2015). A basement membrane was present, delineating the lymphoid accumulation in the remaining gill, similar to the ILT described by Haugarvoll et al., (2008) in Atlantic salmon. Basement membranes are very important for tissue and organ morphogenesis and the maintenance of their functions (Rowe and Weiss, 2008). Additionally, they have a role in protecting the tissues from disruptive physical stresses and mediation of signals from cells to the surrounding environment (Yurchenco, 2011). Basement membranes also play a significant role in cell migration (Rowe and Weiss, 2008). The lymphoid cell accumulation, similarly to the ILT in salmonids, was devoid of blood vessels (Haugarvoll et al., 2008). However, a close association was observed between the afferent filamentary artery and the afferent arterioles in the dILT of Atlantic salmon (Dalum et al., 2015). A similar observation was made about SBT, in which the afferent filamentary artery was surrounded by the lymphoid cell agglomeration and delineated by the basement membrane. In the dorsal sections, several capillaries were observed under the basement membrane that separated the cell accumulation from the adjacent gill structure.

The leading edge of the filament presented a denser epithelial meshwork than the trailing edge. Furthermore, there was a higher density of mucous cells in the leading edge, a lower number of lymphocytes, and denser epithelial meshwork in the leading edge when compared to the trailing edge. The structure of the leading edge was composed of a thicker stromal meshwork than the trailing edge. The fact that the leading edge presented a thick stromal meshwork and many mucous cells may be due to the higher exposition of this area to parasites than the trailing edge (Rodríguez-Marín et al., 2008; Culurgioni et al., 2014) and its susceptibility to physical damage.

Thus, it is possible that the thick stromal meshwork in the leading edge of the filament may reduce physical injury to the leading edge of the filament. In the salmon ILT, the epithelial layer in the trailing edge had more lymphocytes than the leading edge (Dalum et al., 2015b). It was hypothesised that this could be due to the retention time of water, which is longer in the trailing edge, allowing for a close and long interaction between the gill mucosa and the water (Dalum et al., 2015). The same can be hypothesised for the lymphoid tissue described in SBT in the present study.

Studies using T-cell markers in the gills of Atlantic salmon revealed that the majority of the lymphocytes observed were T-cells, a majority of which were CD4+ cells (T-helper cells) that coordinate an array of immune responses including production of cytokines, which has an effect on the communication and interaction of cells (Koppang et al., 2014, Aas et al., 2014, Zhu & Paul, 2008). The lymphocyte morphotype in the lymphoid cell aggregation of SBT was not identified. The lack of cell markers limits the understanding of the cell population in the gills of tunas as well as many other fish species, and most of the information regarding immune cells in tunas is limited to transcriptional analysis (Koppang et al., 2015). In the gills of Pacific bluefin tuna infected with *Cardicola orientalis* and *C. opisthorchis* there was a significant increase of IgM and minimal T-cell involvement and antigen presenting cells in the immune response (Polinski et al., 2014). IgM is a marker for B lymphocytes and plasma cells. However, this isotype is predominantly found in the blood (Secombes and Wang, 2012). Thus, it is unknown if the presence of IgM was due to the involvement of the cell population in the gills of the tuna or a result of a systemic immune response. It has been suggested that, similarly to other blood flukes, those belonging to the genus *Cardicola* may have evolved a strategy against the tuna immune system by suppressing the T-cell response, thus, explaining the low expression of T-cell markers (Polinski et al., 2014). It is likely that a mixed population of lymphocytes forms the lymphoid cell agglomeration, as observed in teleost MALTs (Salinas, 2015). However, further studies with specific cell markers will be required to analyze the lymphoid cell population further in the gills of uninfected SBT. There has been just one attempt to describe an organised MALT in another teleosts species, sea bass (*Dicentrarchus labrax*) (Nuñez Ortiz, et al., 2014). There was a high number of T-cells in the lamellae epithelium in the seabass but no evidence of an organised structure like the ILT.

The lymphoid structure exhibited cytokeratin positive cells with a trabecular pattern. This structure harboured lymphocytes and eosinophilic granular cells, in a similar way to the ILT in Atlantic salmon (Haugarvoll et al., 2008). In the basal area, epithelial cells formed a palisade-like structure.

Mucous cells were present on the surface of the lymphoid structure. A higher density of mucous cells was observed in the leading edge of the filament, with the mucous cells present under the epithelium, when compared to the leading edge, in which the mucous cells presented a single and more dispersed layer. The location of the mucous cells in the ILT of SBT, in the surface of the lymphoid structure, was identical to the one observed in the ILT in Atlantic salmon (Dalum et al., 2015). Furthermore, the difference in the mucous cell density, with a higher density in the trailing edge when compared with the leading edge was also observed in the ILT of salmonids (Dalum et al., 2015).

us cells were apically embedded in the epithelium that limited the lymphoid accumulation, similarly to the salmon ILT. A higher density of mucous cells was observed in the trailing edge of the filament, with the mucous cells present under the epithelium, when compared to the leading edge, in which the mucous cells presented a single and more dispersed layer. The mucous cells in the ILT of Atlantic salmon presented the same pattern as the ones in SBT (Dalum et al., 2015).

A large number of EGs were observed in the gill lymphoid structures of SBT. H&E, PAS and immunohistochemistry for piscidin demonstrated that EGs had different stain affinities. Therefore, it was likely that a mixed EG population was present in the lymphoid structure of the gills of SBT. When observed histologically, there were two types of eosinophilic granular cells (EGCs) that were likely two different populations. One type was strongly eosinophilic, and had a small peripheral nucleus, whereas the other type was weakly eosinophilic and had an elongated and central nucleus. However, it is important to consider that different angles of sectioning may have led to structural differences. Furthermore, regarding the expression of piscidin, it is possible that in the same cell population cells differently express the antibacterial peptide.

The most common eosinophilic granulocytes in teleosts, and possibly present in the cell agglomeration described here, are eosinophilic granular cells/mast cells (EGC/MCs) and eosinophils (Reite and Evensen, 2006). The presence of EGC/MCs in the gills of zebra fish

(*Danio rerio*) and salmonids have been previously described (Powell et al., 1990; Reite, 1997). Furthermore, these cells have been described in the lymphoid organs of some fish species, such as in the head kidneys of zebrafish and European chub (*Squalius cephalus* L.) (Balla et al., 2010; Kondera, 2014). EGC/MCs are frequently in proximity to surfaces that interface with the external environment; in the case of fish, they are most abundant in the gills, gut, and skin (Gomez et al., 2013). EGC/MCs release piscidins and act upon the pathogens (Silphaduang and Noga 2001, Silphaduang et al., 2006). Piscidins are antimicrobial peptides, which act by disrupting the membranes of cells and pathogens, making them a very broad antibacterial (Noga and Silphaduang, 2003). Furthermore, it has been hypothesized that piscidins may also kill pathogens after phagocytosis (intracellularly) (Silphaduang et al., 2006). A previous study showed the presence of MCs in the gills of several species of fish from the suborder percoidei using piscidin antibody (Corrales et al., 2010, Dezfuli et al., 2010 ).Previous research on the ontogeny of Pacific bluefin tuna described the thymus, spleen and anterior kidney from 0.5 to 30 days post hatch (Watts et al., 2003). In none of the stages of development was the presence of EGC/MCs observed. However, the presence of EGC/MCs may differ in late life-stages. The SBT used in this experiment were adults, and it is possible that these cells accumulate in these organs in late life-stages.

EGC/MCs are known to be involved in host response to bacterial and helminth pathogens at mucosal sites, such as gills and gut (Secombes and Chappell, 1996). These cells can degranulate to have a function analogous to mammalian MCs (Secombes and Chappell, 1996). EGC/MCs are known to share some functions and be closely associated with other cells of the immune system, such as eosinophils, neutrophils and rodlet cells (Sfacteria et al., 2014). A previous study of the gills of Atlantic bluefin tuna found a high number of mast cells, eosinophils and some rodlet cells in areas where cysts of *D. katsuwoncola* were present (Pleic et al., 2015). It is possible that the cell accumulation observed in SBT is a mix population composed of eosinophils and mast cells. TEM is a valuable way of differentiating between these cells. However, in the present study, the TEM sections showed the presence of lymphocytes and epithelial cells, but granulocytes were not observed in the sections.

IHC revealed that some of the cells had a strong expression of piscidin 3, whereas others had a scant or negative expression. EGCs/MCs staining in histological sections and distribution among



tissues are highly variable across fish species (Sfacteria et al., 2015). Despite the heterogeneity of EGCs/MCs characteristics in some teleost species, the functions of these cells are very similar to mast cells in mammals. EGCs/MCs are essential as initiators and effectors of innate immunity, and they regulate adaptive immune responses (Mulero et al., 2007). Furthermore, they induce smooth muscle contraction and alter vascular permeability and vasomotor response (reviewed in Sfacteria et al., 2015). Water is an environment with a high prevalence of infectious agents (Bergh et al., 1989), and the respiratory epithelium of the gills are entry points for pathogens. When a mucosal barrier detects a pathogen, an immediate innate immune response is triggered, which will help in the establishment of the adaptive immune response (Salinas, 2015). Thus, it is likely that the EGCs/MCs in the lymphoid structure in the gills of SBT have a surveillance role to enable quick responses to pathogens, limiting possible gill damage. EGC/MCs have an important role in parasitosis (Reite, 1998). Due to their mobile characteristics, they are able to migrate to infected areas (Reite and Evensen, 2006; Dezfuli et al., 2013a). Reite, (1998) suggested that teleost species subjected to a low pathogenic pressure commonly keep few EGC/MCs in stand by and rely on their efficient mobilisation. The high number of EGC/MCs in the gills of SBT corroborate this theory because parasites frequently affect the gills of SBT (Munday et al., 2003; Rough, 2000). The presence of EGCs/MCs in the connective tissue of healthy individuals of brown trout (*Salmo trutta*) and tench (*Tinca tinca*) has been described to be closely associated with blood vessels (Dezfuli et al., 2012a; Dezfuli et al., 2012b). This was also observed in the gills of SBT and may indicate that these cells may not only have a role in the gill immune response but also transit in the capillaries and arteries to other organs. The presence of EGC/MCs has not been previously described in tuna lymphoid structures.

The presence of a lymphoid structure similar to the ILT described in salmonids but with a higher population of eosinophilic granulocytes may indicate an evolution of the tissue. There are few studies in the evolution of cell population of MALTs in fish. However, a previous study in EGCs/MCs revealed that these cells lack histamine, which is a component of mast cells in mammals. However, further studies revealed that the EGCs/MCs of Perciforms, which are the most evolutionarily advanced order of teleosts, contain histamine (Mulero et al., 2007). This may reveal that this cell population may have evolved within teleosts. EGCs/MCs are normally located in proximity to surfaces that interface with the external environment. Thus, these cells are likely to be the first inflammatory cells to interact with pathogens allergens and toxins,

initiating immune responses (Crivellato and Ribatti, 2010). It is likely that the lymphoid structure in southern bluefin tuna represents a more evolved ILT when compared to those in salmonids. However, further studies will be of high relevance to understanding the functions of this tissue, and confirm the cell types, mainly the lymphocytes and the two possible populations of EGCs/MCs.

Some limitations were present in this study, including lack of antibodies to further investigate the cell populations present in the lymphoid accumulation in the gills of SBT. However, the present study presents a comprehensive study of a gill mucosal associated lymphoid tissue, likely an equivalent to the ILT in Atlantic salmon. Further studies aiming the understanding of cell populations in the gill mucosal associated lymphoid will help understand the cellular immune response in the gills of tuna.

## 4.7 Conclusion

This study describes a novel GIALT in the gills of SBT, composed mainly of lymphocytes and eosinophilic granulocytes, most likely EGCs/MCs. Its location and structure suggest that it may be the equivalent of the salmonid ILT in tuna. To the best of my knowledge, this is the first time a GIALT with a high population of eosinophilic granulocytes has been described in tuna. The investigation of this tissue may generate valuable information in developmental immunology, and may lead to further immunological studies in farmed tunas.

# Chapter Five

## General Discussion

### 5.1 Effects of AGD in the ILT

The results of this PhD research are the first to show that the ILT of Atlantic salmon is plastic in terms of its response to an economically important marine parasite, *N. perurans*, the causative agent of AGD. When this study was planned, it was unknown that dorsal sections were so helpful to analyse the dILT. However, analysing the dILT in transversal sections may lead to errors, due to be in a larger area, in comparison with the pILT, in which the location is fixed, and when performing the analysis, it is certain that the area analysed is the same in all the fish. Thus, the pILT was used since it is the most constant area of the ILT (location wise). Chapter 2 of this research, which investigated the effects of a single *N. perurans* exposure, showed that AGD induced significant morphological changes and a cellular response in the ILT of affected fish. These changes included an increase in the ILT surface area and length at 28 days post exposure. Immunohistochemistry revealed that epithelial hyperplasia was the most likely factor contributing to the ILT enlargement in the affected fish. Epithelial hyperplasia in the gills is well documented as a pathology of AGD lesions (Adams and Nowak 2003) however this is the first time it has been documented in the ILT. In this study, it was hypothesised that lymphocytes were possibly migrating from the ILT to lesion sites due to the observation of lower numbers of lymphocytes (Chapter 2) or the observation of the diffusion of CD3<sup>+</sup> cells from the dILT to lesions (Chapter 3). Lymphocyte migration resulting in a reduction of the size of the ILT has been suggested for fish infected with the ISA-virus (Aas et al., 2014). Thus, it is possible that T-cells in the ILT migrate to areas of infection when needed and that the ILT can act as a storage of immune cells during infection or challenge. The retention of T and B lymphocytes in mucosal surfaces in mammals has been well described, and it is divided in two specific sites: inductive sites, where antigens are sampled from mucosal surfaces and stimulate cognate T and B-cells and effector sites, in which the effector cells complete their action (such as formation of secretory IgA) (Brandtzaeg et al., 2011). Although the current knowledge of teleost mucosal structures is limited it is believed that MALTs in teleosts also undertake both functions (Salinas, 2015). As

the ILT is considered an organized MALT, further studies on migration, differentiation and function of mucosal B and T cells in the ILT will contribute to our understanding of the functions of the ILT and MALTs in fish.

ILT plasticity in response to a marine parasitic infection was a key finding in this research. The ILT plasticity and responsiveness to infection highlights the potential for future studies regarding fish mucosal immunity and new avenues of disease management and treatment strategies particularly for cultured salmonids and AGD. A more in depth knowledge of the ILT will shed new light on the mucosal immune system in the gills, and can be of high importance to the development of cost-effective vaccines in aquaculture (Koppang et al., 2015). One of the most efficient delivery methods is bath immunization, since the large area of the gills optimize vaccine uptake (Koppang et al., 2015). Thus, understanding of the gill mucosal immunity will help in the designing of effective vaccines and delivery systems.

In this PhD research, it was observed that the dILT was often modified when in close proximity to AGD lesions (Chapters 2 and 3). Immunohistochemistry showed that when lesions presented a high number of CD3<sup>+</sup> cells, there was often a high number of CD3<sup>+</sup> cells in the dILT adjacent to the lesions. In some sections, a diffusion of CD3<sup>+</sup> cells from the dILT to the lesions was observed. CD3<sup>+</sup> cell infiltration was observed in some lesions and it is likely that these cells were migrating from the ILT to the lesions. Lymphocytes migrate within the immune system, and in humans, their circuits are well established (Mikhak et al., 2015). Lymphocyte trafficking plays a very important role in homeostasis, host defence and pathogenesis (Mikhak et al., 2015). Lymphocyte migration also occurs in teleosts, and substantial research has been done on zebrafish, which have been used as a model for mammal lymphocyte migration (Deng and Huttenlocher, 2012). With advances in the aquaculture industry, studies in fish mucosal immunity, including cell migration, have focused on species important to aquaculture (Castro and Tafalla, 2015). Despite the contributions of the present research, additional studies are required to further understand the kinetics and roles of lymphocytes in the ILT and adjacent lesions. Some AGD lesions observed in this study did not present infiltration of CD3<sup>+</sup> cells, and the dILT close to the lesions had low or zero CD3<sup>+</sup> cells and was frequently hyperplastic. It is possible that the lesions observed were representative of different infection stages of AGD. The variability in AGD severity is very high, and the most explored factor is individual genetics,

leading to selective breeding for AGD resistance (Kube et al., 2012). The factors that may contribute to the variability of AGD severity, including the unsynchronised variability in individuals in the water column (Oppedal et al., 2011), the distribution of *N. perurans* in the water column (Wright et al., 2015), or an interaction of both factors. Studies on parasitic epidemiology indicate that, in spatially heterogeneous populations, hosts have a different rate of exposure to the parasite compared to spatially homogeneous populations (Frank, 2002). Heterogeneous populations may uphold temporary isolated refuges in which hosts have low exposure to the parasite (Frank, 2002). This can be true in the experimental tanks, in which some individuals may be exposed to *N. perurans* later than others and, thus, infected at a later stage. Therefore, it is important to consider that individual fish from the same cohorts (in the same experimental tanks) may have been exposed to *N. perurans* at different times, and the differences observed may have resulted from this factor.

The Atlantic salmon used for the analysis of the ILT during AGD were from experimental AGD trials. The use of naïve salmon exposed to *N. perurans* provided indirect evidence of innate cell immune response. Some fish that were collected at 14 and 28 days post-exposure presented severe gross pathology, and when observed histologically, the ILT had significant morphological alterations (see Chapter 2). Salmon repeatedly exposed to *N. perurans* were used, simulating AGD in a production environment where fish are bathed and re-infected several times. These fish presented less severely affected gills and no extreme changes in the ILT morphology were present like the ones observed in Chapter 2. Differences in results between Chapters 2 and 3 highlight the complexity of not only AGD but also the ILT itself. As discussed various factors can significantly affect AGD progression (Oppedal et al., 2011; Wright et al., 2015; Frank, 2002) and several studies have been performed to understand the source of AGD severity variability (Taylor, Wynne et al. 2007; Taylor, Kube et al. 2009; Kube, Taylor et al. 2012). AGD is a complex disease and differences in pathology among similarly infected individuals is commonly observed at both a gross and histological level (Taylor et al., 2009). These results highlight the complexity of understanding the ILT under disease conditions however the ILT plasticity and observed cellular changes consistently provide support for further study of this structure due to the potential for treatment avenues.

## 5.2 Southern Bluefin Tuna Organised GIALT

As well as a target for mucosal immunity studies the ILT in salmonids is regarded as an important structure for evolutionary studies as it the only organized MALT identified in fish. An additional aim of this research was to determine if a gill mucosal associated lymphoid tissue (GIALT), similar to the ILT in salmonids, was present in another commercially valuable teleost species, the SBT. In this study a GIALT in SBT was located in the same area of the ILT in salmonids. Similar to the ILT, the GIALT in tuna was composed mainly of lymphocytes embedded in a meshwork of epithelial cells and a high number of eosinophilic granulocytes (Figure 5. 1). When analysed histochemically, it was observed that the eosinophilic granulocytes were possibly from two different distinct populations (Chapter 3). Further studies, perhaps focusing on molecular techniques will be required to correctly differentiate these cell types. Although not currently available, southern blue fin tuna specific B and T-cell markers would also be highly useful to identify lymphocyte populations observed in the tuna GIALT. The organised GIALT observed in tuna extended along the trailing edge of the filament and was also present in the leading edge and in the lamellar fusion.

There has been just one attempt to describe an organized MALT in another teleosts species, sea bass (*Dicentrarchus labrax*) (Nuñez Ortiz, et al., 2014). There was a high number of T-cells in the lamellae epithelium in the seabass, but no evidence of an organised structure like the ILT. During this PhD project, the gills of several fish species such as yellowtail kingfish (*Seriola lalandi*), southern sand flathead (*Platycephalus bassensis*) barramundi (*Lates calcarifer*) and mullet (*Mugilidae sp.*) were observed in an attempt to find an organised structure, like the ILT described in salmonidss. In none of the species was there a clear presence of a lymphoid structure observed. However, caution is needed when observing the gills of fish. Gills are diverse among teleosts and looking for a structure in the interbranchial septum may not be feasible in other species due to the degree of gill septum development which is diverse among fish species (Hughes, 1984).

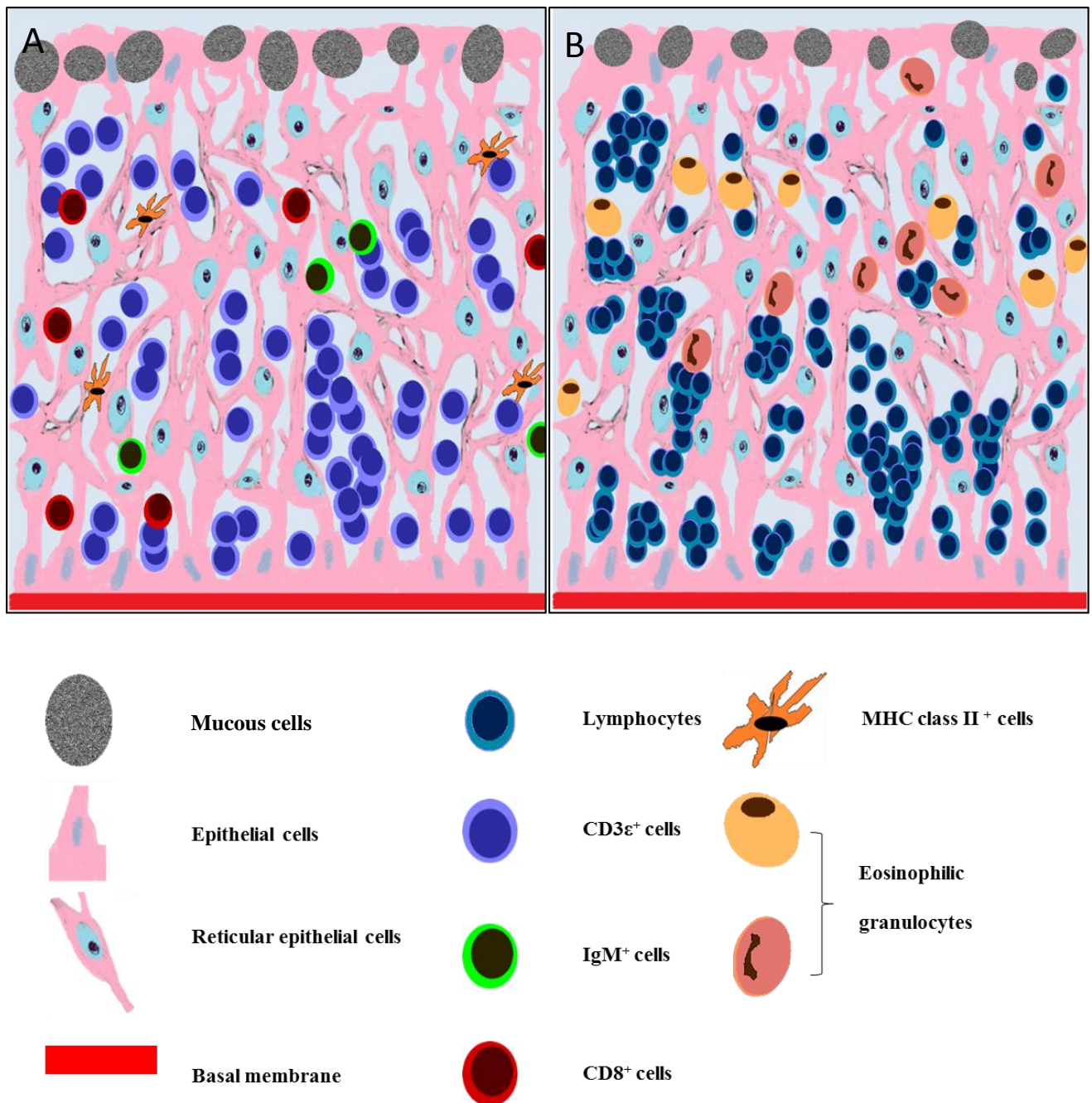


Figure 5. 1 Comparison of the structure of the (A) salmonid ILT with (B) tuna GIALT. In both lymphoid tissues, the superficial layer was composed of mucous cells and epithelial cells. The medial structure in the ILT is composed mostly by CD3<sup>+</sup> cells embedded in a meshwork of epithelial reticular cells. In the tuna GIALT, lymphocytes are present, and two morphologically distinct eosinophilic granulocytes are common cells in the structure. MHC class II<sup>+</sup> cells are present in the ILT. It is unknown if antigen presenting cells are present in the GIALT.

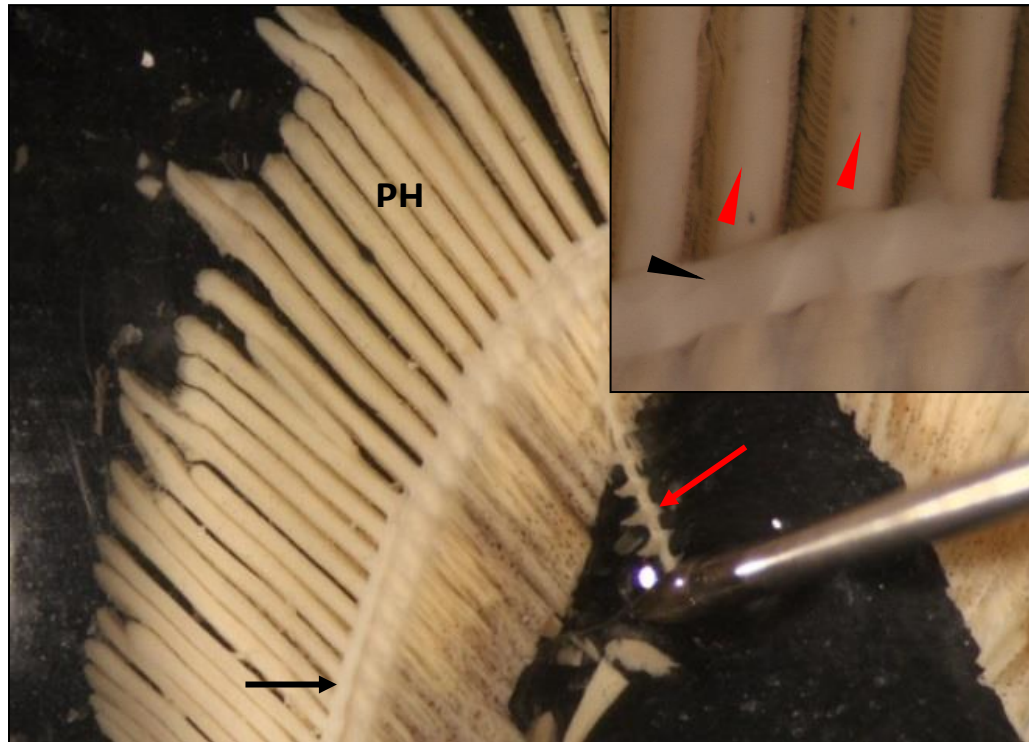
## 5.3 Key Future Directions

The observation of differences in the number of lymphocytes in the ILT associated with AGD lesions suggests that lymphocyte trafficking occurs in the ILT. Research in lymphocyte trafficking in humans has led to the identification of several novel therapeutic targets and, consequently, the development of new therapies, including the development of therapeutic regimens for asthma (Mikhak et al., 2015). Asthma is a chronic inflammation of the airways with activation and infiltration of inflammatory cells, mainly Th2 lymphocytes and eosinophils (Mathew et al., 2002). Inhibition of lymphocyte trafficking in asthma has been attempted and is in the clinical trial phase by blocking the C-C Motif Chemokine Receptor 4 (CCR4), which will disrupt the trafficking of Th2 cells to the airways (Barnes, 2016). It has been found that during AGD, the Th2 pathway was significantly up-regulated (Benedicenti et al., 2015). It is possible that the T-cells of the ILT, which are mainly T-helper cells (CD4<sup>+</sup> cells) are recruited to the lesions, thus, exacerbating the inflammation in the gills (Aas et al., 2014). A further understanding of lymphocyte trafficking, cell population and kinetics during AGD may lead to the development of therapies that are more feasible than freshwater baths.

Transcriptional analysis of the ILT in fish affected by AGD is also a key step in furthering our understanding of ILT function during disease. Limited time, primers and the costs of analysis prohibited this work in this study. Previous studies that aimed to analyse the expression of immune related genes in the gills of Atlantic salmon affected by the ISA-virus used laser capture microdissection to isolate the ILT from the remaining gill tissue (Austbo et al., 2014). It was demonstrated that the ILT presented a moderate immune response to the ISA-virus, but no replication of the virus was detected in it. Thus, it was suggested that the immune response was triggered by antigens presented by local antigen presenting cells, or it was a result of systemic response (Austbo et al., 2014). Further studies revealed that TCR $\alpha$  transcript in the ILT of ISA-virus infected fish decreased following infection, which was a different trend from the transcripts observed in the gills (Austbo et al., 2014). The differences in the immune transcripts between the ILT and the gills reveal that it is important to study these structures independently. Laser microdissection requires specialised equipment that is not available in every lab. A technique attempted during this PhD project to successfully separate the ILT consisted of removing the filaments of the anterior hemibranch using forceps under a dissection microscope (Figure 5. 2).



Using this technique, it was possible to separate the proximal ILT from the rest of the gill. The separation of the dILT from the gill filament presents a more difficult task as the dILT is present in the both the trailing and leading edge of the filaments. Future analysis of the ILT using gill filaments as comparative controls should aim to exclude the edges of the filament if possible.



*Figure 5. 2. Atlantic salmon gill arch with the anterior hemibranch removed, revealing the posterior hemibranch (PH) and the ILT (black arrow), which was removed using forceps (red arrow). Insert shows a closer view of the posterior hemibranch with the proximal ILT (black arrowhead) and dILT (red arrowheads).*

Future studies should also incorporate additional antibodies in order to further analyse the major population CD3<sup>+</sup> cells in the ILT, such as CD4 (targeting T-helper cells) and CD8 (cytotoxic T-cells), which have been previously observed in salmon ILT. Further analysis of B-cells could also be of interest in the ILT during AGD, to observe if antibodies are present in this tissue. Previous research suggested that clonal expansion of IgT expressing B-cells occurred in the ILT of Atlantic salmon infected with ISA-virus, in the latest phases of the infection (Austbo et al., 2014).

## 5.4 Conclusion

The ILT is regarded as an important structure both for possible development of mucosal vaccines, and also evolutionary studies as it is the only organized MALT known in teleosts. However, little is still known, and the majority of the studies focus on transcriptional analysis. This research aimed to understand the effects of a protozoan parasite, *N. perurans* on the ILT. The use of histopathology and immunohistopathology have shown that the ILT is a plastic structure which is modified by AGD. AGD is a significant health problem for marine aquaculture worldwide (Bustos et al., 2011; Crosbie et al., 2010; Mouton et al., 2014; Munday et al., 2001) and there is a significant economic drive for greater management and treatment options. The results of this work, which provide greater insight into immune modulation at the level of the gill, will benefit aquaculture industries in terms of highlighting the ILT as a novel target for disease management and treatment strategies research. In addition, this research has described a novel GIALT in southern bluefin tuna, another economically important cultured species. This novel structure in tuna similarly can be used for mucosal immunity studies but also offers the study of the immunity in teleosts from a phylogenetic and evolutionary point of view.

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